



UNIVERSIDADE DA BEIRA INTERIOR
Ciências da Saúde

Preclinical performance of vaginal semisolid products: technological and safety evaluations assuming physiologic parameters

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To my Family - À minha Família

“Tenho em mim todos os sonhos do mundo.”

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Resumo alargado

A avaliação pré-clínica de produtos semi-sólidos de administração vaginal quando é realizada tendo em consideração as particularidades intrínsecas desta via de administração, pode constituir-se como um ponto-chave na previsão do desempenho *in vivo* destes produtos. De facto, os medicamentos semi-sólidos vaginais são largamente prescritos e utilizados pelas mulheres, sendo também a classe de formas farmacêuticas de administração vaginal preferida por estas. Antes de entrarem nas fases clínicas do desenvolvimento, os semi-sólidos vaginais devem ter previamente demonstrado um perfil tecnológico adequado e devem ter dado provas de serem seguros. Deste modo, será possível obter maiores taxas de sucesso nas fases seguintes: pré-clínicas em animais (*in vivo*) e clínicas em humanos. Os métodos compendiais tradicionais de caracterização galénica e tecnológica não consideram as especificidades da via de administração vaginal, uma vez que não têm em conta, por exemplo, o pH vaginal, a composição e volume do fluido vaginal e a temperatura corporal. Além disso, mesmo na literatura científica é escassa a informação sistematizada de quais os parâmetros significativos a avaliar neste tipo de formulações. Avaliações precoces dos perfis de segurança e eficácia de medicamentos, são amplamente aconselhados, e, embora poucas, algumas estratégias e métodos encontram-se já descritas e validadas por agências reguladoras, contudo, concentram-se principalmente em modelos celulares. Adicionalmente, se por um lado a segurança pode ser abordada em testes de toxicidade, por outro, pode ainda ser mais amplamente caracterizada através da conjugação com resultados provenientes de estudos de libertação e permeação de fármacos. É um facto que a via de administração vaginal permite não só a administração de fármacos com vista a efeitos localizados, mas também efeitos sistémicos. Sendo que, uma ou outra pode ser desejada e a forma farmacêutica deve permitir que o fármaco seja confinado ao local de administração pretendido, para que se obtenha a máxima eficácia, evitando os efeitos secundários. O objetivo deste trabalho consistiu na realização de uma avaliação pré-clínica completa em formulações já comercializadas, a fim de estabelecer novas abordagens metodológicas que possam ser facilmente aplicadas no desenvolvimento e caracterização de novos produtos.

Neste contexto, medicamentos semi-sólidos vaginais antimicrobianos, tais como, o Gino-canesten®, Sertopic®, Dermofix®, Gyno-pevaryl®, Lomexin®, Gino Travogen®, Dalacin V®; estrogénios, como o Ovestin®, Blissel® e Colpotrophine®; e ainda duas formulações de referência, o Placebo Universal e o Replens® foram amplamente avaliadas. Em termos tecnológicos foram testados o pH, a capacidade tampão, a osmolalidade, alguns parâmetros de textura e viscosidade, seguindo um ponto de vista fisiológico, em que se consideraram a temperatura corporal e a diluição num simulante de fluido vaginal (SFV). Foi ainda otimizado, um modelo vaginal suíno *ex vivo* para estudar estas formulações no que respeita à bioadesão e à reologia num ambiente pós-administração. Quanto ao estudo da segurança, a toxicidade

celular foi avaliada nas linhas celulares: VK2 E6/E7 (vaginais), HeLa (cervicais) e HEC-1A (uterinas), e para tal, foram realizados os ensaios de viabilidade que utilizaram o MTT (brometo de 3-(4,5-dimetiltiazol-2-il)-2,5-difenil tetrazólio) e o NRU (captação de vermelho neutro). Além disso, tecidos epiteliais *ex vivo*, foram excisados a partir da vagina suína, e nestes, também foi aplicado o ensaio do MTT e, ainda uma análise histológica. Para incluir uma avaliação ainda mais avançada da toxicidade *in vitro*, os semi-sólidos vaginais foram testados num modelo organotípico: HET-CAM (ensaio que é realizado na membrana corio-alantóide de embriões de galinhas Henn) e que se encontra em validação para substituir o teste *in vivo* em animais para testes de irritação ocular. Deste modo, foi transposto este ensaio para a avaliação da irritação vaginal. Um método de quantificação de HPLC-DAD (Cromatografia Líquida de Alta Pressão acoplada a um detetor de díodos) para as moléculas presentes nos semi-sólidos vaginais incluídos neste trabalho, foi desenvolvido e validado de acordo com os requisitos das normas da FDA (Food and Drug Administration - EUA), da EMA (Agência Europeia de Medicamentos) e da ICH (Conferência Internacional para a Harmonização). Depois disso, este método foi aplicado na quantificação de fármacos nos estudos de libertação *in vitro* e nas experiências de permeação de fármacos *ex vivo*. Estas duas técnicas foram realizadas utilizando células de difusão dinâmicas verticais de Franz, tendo todas as configurações experimentais sido especificamente otimizadas para as moléculas em estudo (estriol, clotrimazol, econazole, isoconazol, sertaconazol e fenticonazol).

Quanto às características tecnológicas, as formulações antimicrobianas apresentaram menor pH do que os estrogénios tópicos. A capacidade tampão no SFV conduziu a melhores previsões do que acontece *in vivo*. Após diluição em volumes fisiológicos de SFV, a osmolalidade da maioria dos produtos esteve conforme o recomendado pela Organização Mundial da Saúde (OMS), claramente dependente da composição. Os antimicrobianos apresentaram comportamentos de textura semelhantes entre eles, enquanto os estrogénios tópicos variaram entre eles, no que respeita aos parâmetros de textura. Com efeito, neste grupo encontrava-se uma formulação de base polimérica em gel (Blissel®), sendo que os restantes, eram cremes (Ovestin® e Colpotrophine). Regra geral, para todos os produtos observou-se uma ligeira diminuição da viscosidade após aplicação de fatores fisiológicos, como a diluição em SFV e a temperatura (37°C), mas os seus comportamentos pseudoplásticos mantiveram-se. No entanto, cada formulação demonstrou ter o seu próprio perfil reológico, possivelmente conduzido pela sua composição, tanto qualitativa como quantitativa. Contudo, a viscosidade das formulações foi mais elevada quando testada num modelo de administração *ex vivo*, comparando com a viscosidade obtida nos ensaios em SFV a 37°C. Este dado releva, que podem existir outros fatores determinantes na viscosidade adquirida após a administração de formulações semi-sólidas. Quanto aos modelos *in vitro*, as células VK2 E6/E7 apresentaram viabilidades relativamente superiores às células HeLa e HEC-1A nas concentrações de produto testadas. Os resultados de viabilidade no tecido foram muito superiores aos obtidos para nos modelos celulares. Isto revela que este modelo poderá ser mais robusto e mais próximo do que acontece *in vivo*. Em todos os modelos, as formulações antimicrobianas mostraram viabilidades

dependentes da concentração. Ao contrário das formulações que contém estrogénios, que apresentaram perfis relacionados com os efeitos celulares estrogénicos reconhecidos (ou seja, foram dependentes da dosagem da formulação e da concentração testada). Os produtos de referência apresentaram os perfis de viabilidade mais estáveis e mais elevados em todas as concentrações. Nos estudos de permeação *ex vivo*, foi questionada a existência de diferenças na utilização de secções de tecido vaginal caudal ou cranial. Não obstante, em termos de permeação de fármacos, não foram encontradas extensas diferenças significativas entre estes dois tipos de epitélio, mas a vagina caudal parece ser mais adequada para experiências de permeação vaginal, uma vez que conduz a resultados mais reprodutíveis e consistentes. De facto, a região mais cranial da vagina suína apresenta, histologicamente, um epitélio mais fino, e anatomicamente, uma superfície que contém mais rugas. O que conduz a desvios-padrão entre experiências mais elevados. Além disso, observou-se que a permeação de fármacos não é apenas dependente da libertação do fármaco a partir formulação. A sua afinidade pela formulação e/ou pelo tecido, é determinante para a sua permeação até à câmara recetora.

Concluindo, a avaliação pré-clínica integrada para semi-sólidos vaginais, proposta nesta tese, representa uma abordagem importante a ter em consideração, no desenvolvimento de novos produtos, uma vez que pode reduzir custos com o desenvolvimento de novas formulações, prevendo precocemente a sua eficácia *in vivo* e os seus perfis de segurança. Estas metodologias têm, assim, um grande potencial não só para serem aplicadas na indústria cosmética, farmacêutica e de dispositivos médicos, mas também pode ser aplicada em investigação mais fundamental ao nível da academia.

Palavras-chave

Administração vaginal de fármacos, semi-sólidos vaginais, avaliação pré-clínica, caracterização tecnológica, *in vitro*, *ex vivo*, segurança, permeação de fármacos, libertação de fármacos

Abstract

Vaginal semisolid products preclinical evaluations, when performed considering the particularities of the target organ, may represent key tools to predict *in vivo* performance. Before heading to clinical phases, vaginal semisolids must demonstrate to have an adequate technological and safety profile, in order to achieve higher success rates in the human testing stage. Traditional characterization methods currently used for vaginal semisolids do not undertake an integrative approach, since they do not address, for example, vaginal pH, fluid and temperature. Moreover, early safety assessment methods are largely described and validated not only on scientific literature, but also by regulatory agencies, although they are still mainly focused on cellular-based models. This safety profile of products can be further improved by combining toxicity testing, with drug release and permeation studies. Indeed, the vaginal administration route allows for local and systemic delivery of drugs, depending on the therapeutic purpose. Consequently, the drug should be confined to the chosen location of administration, to obtain maximum efficacy while avoiding side effects. The aim of this work was to develop a full set of assessment methods for characterization of vaginal semisolid products. Commercialized formulations were used to establish new methodological approaches that could be applied in new products development and characterization.

Therefore, antimicrobials, Gino-canesten®, Sertopic®, Dermofix®, Gyno-pevaryl®, Lomexin®, Gino Travogen®, Dalacin V®; oestrogens, Ovestin®, Blissel® and Colpotrophine®; and, two reference formulations, Universal Placebo and Replens® were extensively evaluated. Technologically, they were tested in terms of pH, pH-buffering capacity, osmolality, textural parameters and viscosity, using a physiologic standpoint that considered the body temperature and dilution in a physiologic volume of vaginal fluid simulant (VFS); and even an *ex vivo* porcine model to infer bioadhesion and rheology on an after-administration environment. In terms of safety investigation, cellular toxicity was disclosed on VK2 E6/E7, HeLa and HEC-1A cell-lines, using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and NRU (Neutral Red Uptake) assays. Tissue explants, collected from the *ex vivo* porcine vaginal model, were also tested concerning toxicity, through MTT and histological analysis. Moreover, to include an advanced *in vitro* toxicity evaluation, the HET-CAM (Hen's Egg Test - Chorioallantoic Membrane), already in validation for eye irritation testing, was applied to vaginal irritation. A HPLC-DAD (High Performance Liquid Chromatography with Diode Array Detector) quantification method for the molecules present in semisolids included in this work, was developed and validated according to FDA (Food and Drug Administration - USA), EMA (European Medicines Agency) and ICH (International Conference for Harmonization) requirements. Further, this method was applied in drug quantification on *in vitro* drug release and *ex vivo* drug permeation experiments. These two techniques were performed using dynamic vertical Franz diffusion cells, having all experimental setting being specifically designed and optimized concerning the

molecules in study (estriol, clotrimazole, econazole, isoconazole, sertaconazole and fenticonazole).

Concerning technological characteristics, antimicrobial formulations exhibited lower pH than topical oestrogens. Buffering capacity in a vaginal fluid simulant conducted to better predictions of what happens *in vivo*. Characterization was performed also for those less acidic products to assess their ability to gain physiologic pH after mixing with simulated vaginal fluids. Products osmolality after dilution in VFS were below the upper limit advised by the World Health Organization (WHO). The antimicrobials had similar textural behaviours, while topical oestrogens varied in textural parameters. A slight decrease in viscosity was observed after application of dilution and temperature factors, showing the influence of the surrogate vaginal environment, while maintaining their pseudoplastic behaviour. However, each formulation had its own profile, possibly driven by their composition. Formulations' viscosity was higher when tested using the *ex vivo* administration model than when only diluted in VFS at 37°C. Concerning the *in vitro* models, VK2 E6/E7, presented relatively higher viabilities than HeLa and HEC-1A cells over the tested product concentrations. Tissue viability results were much higher than those obtained for the *in vitro* cellular models, revealing that this model could be more robust and closer to the *in vivo* situation. Across models, antimicrobials showed concentration-dependent viabilities. While oestrogens presented odd profiles, depending on the formulation and concentration tested. Reference products led to the most stable and higher viability profiles across concentrations. On *ex vivo* permeation studies we have investigated if there were differences in performing *ex vivo* permeation studies using the porcine vaginal model, when collecting a proximal or a distal tissue within the vaginal tube. No extensive significant differences between these tissues were found, but the caudal vagina could be more suitable for vaginal permeation experiments since it conducted to more reproducible and consistent results. Furthermore, it was observed that drug permeation is not directly dependent on drug release from the formulation.

To sum up, the conduction of this integrative preclinical assessment for vaginal semisolids can be a valuable approach in new products development or characterisation, since it could optimize cost-efficiency of new formulations development by predicting *in vivo* efficacy and safety profiles. In addition, these methodologies have great potential not only to be applied in cosmetics, medical devices and medicines industry, but also in academical research.

Keywords

Vagina drug delivery, vaginal semisolids, preclinical evaluation, technological characterization, *in vitro*, *ex vivo*, safety, drug permeation, drug release

Thesis overview

This doctoral thesis is structured in eight chapters and appendices, herein summarized:

CHAPTER I consists on a brief introduction over the vaginal environment, the vaginal drug delivery systems focusing on semisolids and the characterization methods applied for these fomulations. At the end of this chapter the main objectives of this thesis are presented.

CHAPTER II provides a methodologic background for vaginal semisolid formulations, and, consequently, proposes adaptations to traditional characterization methods, based on results obtained experimentally.

CHAPTER III comprises the development of *in vitro* and *ex vivo* models. A preclinical safety characterization was accomplished through cell-based and organ-based testing.

CHAPTER IV embraces the topic of drug release and permeation. Primarily, a chromatographic quantification method for all drugs included in this study was validated.

CHAPTER V discusses and integrates all the results obtained presented in the previous chapters.

CHAPTER VI describes the contributions that the results of this thesis had for enhancing the competitiveness of the hosting company, Labfit-HPRD, Lda.

CHAPTER VII presents the conclusions of this thesis and future remarks.

CHAPTER VIII details the bibliographic references used to construct this thesis.

APPENDICES contain information that, despite being important for the development of this work, were not essential in the main manuscript. They are composed of: A) a published review article comprising methodologies for vaginal drug permeation; B and C) two manuscripts in the format that was submitted concerning *in vitro* and *ex vivo* toxicity evaluations; D) supplementary material that was gathered to support the experiementsal setup of Chapter IV.

List of Publications

Publications in Peer-Reviewed International Scientific Journals included in this thesis

Machado RM, Agonia AS, Borges L, Palmeira-de-Oliveira A, Martinez-de-Oliveira J, Palmeira-de-Oliveira R, “*Vaginal drug delivery: in vitro release and ex vivo permeation of six active pharmaceutical ingredients from commercial semisolids, quantified by a sole HPLC-DAD validated method*”

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Palmeira-de-Oliveira R, **Machado RM**, Palmeira-de-Oliveira A, Martinez-de-Oliveira J, “*Testing vaginal irritation with the HET-CAM assay: an in vitro alternative to the in vivo method*”

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Machado RM, Palmeira-de-Oliveira A, Breitenfeld L, Martinez-de-Oliveira J, Palmeira-de-Oliveira R, “*Optimization and application of in vitro and ex vivo models for vaginal semisolids safety evaluation*”

(submitted manuscript)

Machado RM, Palmeira-de-Oliveira A, Martinez-de-Oliveira J, Palmeira-de-Oliveira R, “*Vaginal semisolid products: Technological performance considering physiologic parameters*”. European Journal of Pharmaceutical Sciences. 2017; doi: 10.1016/j.ejps.2017.09.009

Machado RM, Palmeira-de-Oliveira A, Silva Gaspar C, Martinez-de-Oliveira J, Palmeira-de-Oliveira R, “*Studies and methodologies on vaginal drug permeation*”, Advanced Drug Delivery Reviews. Special Issue: Vaginal Drug Delivery. 2015; 92:14-26; doi: 10.1016/j.addr.2015.02.003

Other publications during the PhD Course

Machado RM, Palmeira-de-Oliveira R, Agonia AS, Martinez-de-Oliveira J, Palmeira-de-Oliveira A, “*Development and characterization of a new Vaginal Estriol Cream (VEC) for vaginal atrophy - a biocompatible and bioadhesive approach*”

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Palmeira-de-Oliveira R, Palmeira-de-Oliveira A, **Machado RM**, Elvas AR, Amorim I, Gartner F, Martinez-de-Oliveira J, Amaral MH, Breitenfeld L, “*Porcine vaginal model for drug permeation: new insights*”

(submitted manuscript)

Machado RM, Palmeira-de-Oliveira A, Martinez-de-Oliveira J, Palmeira-de-Oliveira R, “*Development and preliminary characterization of a new vaginal dosage form: a vaginal sheet*”

(submitted manuscript)

Palmeira-de-Oliveira R, Macedo M, **Machado RM**, Pacheco AF, Palmeira-de-Oliveira A, Martinez-de-Oliveira J, Duarte P, “*Pharmaceutical Compounding in Portuguese Community Pharmacies: Characterization and Future Perspectives*”, International Journal of Pharmaceutical Compounding, 2016; 20(2):114-122

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Machado RM*, Palmeira-de-Oliveira A, Martinez-de-Oliveira J, Palmeira-de-Oliveira R, *“The porcine ex vivo model for vaginal drug permeation: being more selective”*, XII Annual CICS Symposium, Covilhã, Portugal (2017)

Machado RM*, Palmeira-de-Oliveira A, Martinez-de-Oliveira J, Palmeira-de-Oliveira R, *“Pre-validation of an ex vivo model for vaginal formulations safety evaluation”*, II International Congress in Health Sciences Research towards innovation and entrepreneurship: Trends in Biotechnology for Biomedical Applications, Covilhã, Portugal (2017)

Machado RM*, Palmeira-de-Oliveira A, Martinez-de-Oliveira J, Palmeira-de-Oliveira R, *“Design of innovative vaginal semisolid formulations: physiologic characterization methods as a starting point”*, EUPAT 8 - EUFEPS, University College Cork, Ireland (2016)

Machado RM*, Palmeira-de-Oliveira A, Martinez-de-Oliveira J, Palmeira-de-Oliveira R, *“Physiologic characterization as a starting point towards the design of innovative vaginal products”*, XI Annual CICS Symposium, Covilhã, Portugal (2016)

Machado RM*, *“1 slide, 3 minutes - Your PhD thesis”*, University of Beira Interior, Covilhã, Portugal (2015)

Machado RM*, *“Professional Pathways on Pharmaceutical Sciences - Research”*, UBIPharma Career Opportunities Day, Covilhã, Portugal (2015)

Palmeira-de-Oliveira R*, Palmeira-de-Oliveira A, **Machado RM**, et al., *“Development and Characterization of Vaginal Probiotic Films”*, Pharmabiotics Conference, Paris, France (2014)

Machado RM*, Palmeira-de-Oliveira R, Gaspar C, et al., *“Development and characterization of a new Estriol Cream for vaginal atrophy”*, IX Annual CICS Symposium, Covilhã, Portugal (2014)

Cunha AR*, Palmeira-de-Oliveira A, **Machado RM**, et al. *“Technological characterization of commercially available vaginal products: a safety perspective”*, IX Annual CICS Symposium, Covilhã, Portugal (2014)

Poster communications presented during the PhD Course

Machado RM, Palmeira-de-Oliveira A*, Martinez-de-Oliveira J, Palmeira-de-Oliveira R, *“Formulation, technological characterization and in vitro toxicity of a new vaginal estriol cream (VEC), 2nd ISIDOG CONGRESS, Vienna, Austria (2017)*

Machado RM, Palmeira-de-Oliveira A*, Martinez-de-Oliveira J, Palmeira-de-Oliveira R, *“Vaginal semisolid products: technological performance and in vitro evaluation under a safety perspective”, 2nd ISIDOG CONGRESS, Vienna, Austria (2017)*

Machado RM*, Palmeira-de-Oliveira A, Martinez-de-Oliveira J, Palmeira-de-Oliveira R, *“Predicting vaginal drug delivery: an ex vivo model for rheological behaviour evaluation after administration”, XII Annual CICS Symposium, Covilhã, Portugal (2017)*

Machado RM*, Palmeira-de-Oliveira A, Martinez-de-Oliveira J, Palmeira-de-Oliveira R, *“Preclinical evaluation of vaginal semisolid products: development, optimization and analysis of methods considering physiologic parameters”, Encontro Ciência 2017, Lisbon, Portugal (2017)*

Machado RM*, Palmeira-de-Oliveira A, Martinez-de-Oliveira J, Palmeira-de-Oliveira R, *“Optimization and application of in vitro and ex vivo models for vaginal semisolids safety evaluation”, II International Congress in Health Sciences Research towards innovation and entrepreneurship: Trends in Biotechnology for Biomedical Applications, Covilhã, Portugal (2017)*

Machado RM*, Palmeira-de-Oliveira A, Martinez-de-Oliveira J, Palmeira-de-Oliveira R, *“Design of innovative vaginal semisolid formulations: physiologic characterization methods as a starting point”, EUPAT 8 - EUFEPS, University College Cork, Ireland (2016)*

Batista J, **Machado RM***, Palmeira-de-Oliveira R, *et al.* *“The anti-Candida biofilm effect of Clotrimazole and Trypsin combination tested upon an ex vivo vaginal model”, XI Annual CICS Symposium, Covilhã, Portugal (2016)*

Machado RM*, Palmeira-de-Oliveira A, Costa A, *et al.*, *“Physiologic approaches in characterization methods for vaginal products towards drug delivery optimization, safety and women´s compliance”, 11th BioBarriers, Saarland University, Germany (2016)*

Machado RM*, Palmeira-de-Oliveira R, Gaspar C, *et al.*, *“Cytotoxicity assessment using a commercial in vitro reconstructed human vaginal epithelial model versus an experimental ex vivo vaginal tissue porcine model”, II Iberian Toxicology Journeys, Covilhã, Portugal (2014)*

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List of Abbreviations

A

ABC	Absolute pH-Buffering Capacity
ATCC	American Type Culture Collection

B

BV	Bacterial Vaginosis
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C

CI	Confidence Interval
CLT	Clotrimazole
CV	Coefficient of Variation

D

DAD	Diode Array Detector
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide

E

ECO	Econazole
EDTA	Ethylenediaminetetraacetic Acid
<i>e.g.</i>	<i>exempli gratia</i> meaning “for example”
EMA	European Medicines Agency
EU	European Union
ECVAM	European Centre for the Validation of Alternative Methods

F

FBS	Foetal Bovine Serum
FDA	Food and Drug Administration
FEN	Fenticonazole
F_{\max}	Maximum Force

H

HBSS	Hanks' Balanced Saline Solution
HET-CAM	Hen's Egg Test-Chorioallantoic Membrane
HPLC	High Pressure Liquid Chromatography

I

ICH	International Conference on Harmonisation
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods

i.e.	<i>id est</i> meaning “that is”
ISO	Isoconazole; International Organization for Standardization
L	
LLOQ	Lower Limit of Quantification
LOD	Limit of Detection
M	
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
N	
NS	Normal Saline (NaCl 0.9%)
O	
OECD	Organisation for Economic Co-operation and Development
OTC	Over-The-Counter
P	
PBS	Phosphate Puffer Solution
Q	
QC	Quality Control
R	
RPMI	Roswell Park Memorial Institute
RBC	Relevant pH-Buffering Capacity
S	
SD	Standard-Deviation
SER	Sertaconazole
<i>spp.</i>	Species
U	
USA	United States of America
UV	Ultraviolet
V	
VFS	Vaginal Fluid Simulant
VFS _m	Modified Vaginal Fluid Simulant
VVC	Vulvovaginal Candidosis
W	
W _{ad}	Work of Adhesion
WHO	World Health Organization

CHAPTER I

INTRODUCTORY OVERVIEW

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I.1. Background

Researchers are now devoted to find new forms or to re-discover safer and more effective alternative routes for the administration of drugs that are poorly absorbed orally or suffer precociously metabolism¹⁻³. The vaginal route has been considered of great interest for drug delivery, since it enables both local and systemic drug delivery^{4,5}, allowing for the absorption of peptide and other macromolecules, and even micro and nanoparticles⁶⁻⁸.

The vaginal route provides different advantages over the oral one but it is not deprived of inconvenients^{9,10}. Its large surface area, rich blood supply, ability to bypass hepatic first-passage, avoidance of gastrointestinal side effects, and relatively high permeability to drugs with a wide range of molecular weights drugs are some of its physiological characteristics that contribute to its pharmacokinetic advantages¹¹⁻¹⁴. However, drug absorption through the vagina may be affected by variations of epithelial thickness and by changings in the vaginal milieu composition, that occur as a consequence of age dependent physiological conditions or sexual intercourse; leakage and the self-cleaning action of the vaginal tract may result in low drug bioavailability^{5,15}. Furthermore, general disadvantages of vaginal drug delivery include its obvious gender specificity, cultural background limitations and personal hygienic care interference^{12,14}.

Vaginal drug delivery systems include solutions, semisolids (creams, ointments and gels) and solid formulations (tampons, capsules, pessaries, suppositories, films, sponges, powders and special controlled release devices like the intravaginal ring) as well as other types of formulations such as aerosols and particulate systems integrated in adequate drug delivery systems¹⁶. Efficacy of drug delivery systems will rely on their ability to promote adequate drug concentrations at the targeted site of action, being it local or distant. When a systemic effect is the objective through this route, drugs must be transported across the epithelium to gain access to dermal vessels and the systemic circulation¹⁷. On the other hand, when a local effect is the goal, as is the case for some antimicrobials, microbicides and contraceptive effects, retention of the drug at the surface of the vagina is desirable¹⁸⁻²¹.

Concerning all these particularities added to women's preference and acceptability patterns for vaginal semisolid formulations^{22,23}, a safety- and suitability-driven study can conduct to a better characterization of the already marketed products in the perspective of developing new products.

I.1.1. Historical perspective

The first records of vaginal administration of preparations date back to Ancient Egypt. In the *Kahun Papyrus*, the oldest of medical papyri (ca. 1850 B.C.), refers to vaginal "preparations", which contained natural substances such as mud, frankincense, oil, malachite, ass urine, myrrh, crocodile dung, honey, and sour milk, intended to be used in female genitalia for specific vaginal conditions and contraception²⁴. Some decades after, the *Ramesseum Papyrus* (ca. 1700 B.C.), the *Ebers Papyrus* (ca. 1550 B.C.), and the *Greater Berlin Papyrus* (ca.

1300 B.C.) also described drug formulations to be administered in the vagina. The administration of preparations through the vaginal route was historically succeeded by other civilizations such as Greece, Rome, the Arabic and Oriental cultures, Renaissance and throughout Modern Era until nowadays^{25,26}.

It is believed that primarily the vaginal route of administration was consistently been used for local applications. However, it was only in 20th century that its recruitment for systemic delivery was scientifically hypothesized and studied. Firstly, Macht reported the vaginal absorption of morphine, atrophine and potassium iodide²⁷. In the early twenties, an obstetric surgeon from Westminster and West London Hospitals, conducted an experimental study to determine the vagina's aptitude to absorb some substances. In this study, Robison Drummond concluded that potassium iodide and sodium salicylate solutions were rapidly absorbed and found in urine an hour after being placed in the vagina. Also quinine, cane sugar and phenol red were clinically found to be absorbed and excreted by the kidneys²⁸.

Since then, several drugs have been approved for vaginal administration by medicines' authorities all over the world, the majority being proposed for the treatment of local conditions. In fact, the vaginal is presently considered a well-established alternative administration route to formulations intended both for local or systemic delivery⁴.

I.1.2. Vaginal administration route

Vaginal drug delivery represents an important approach for the treatment of both local and systemic diseases^{1,4,5}. As already stated, the vaginal route has several advantages due to its large surface area, rich blood supply, avoidance of the hepatic first-pass effect, relatively high permeability to many drugs, and allowance of self-insertion¹². These particular characteristics of the vagina provide significant potential for the delivery of a wide range of bioactive compounds, including peptides and proteins, and offer an alternative to the parenteral route of administration¹. In the case of local disease or disorder, using a vaginal product frequently avoids drug absorption in systemically relevant amounts and thus prevents side effects. The vaginal route may be of particular importance in the case of drugs undergoing extensive hepatic metabolism since it avoids the hepatic first-pass effect and allows reducing the amount of administered drugs (e.g. estrogens).

On the other hand, several drawbacks, including cultural background, personal hygiene, gender specificity, local irritation, and influence on sexual intercourse, need to be addressed during the design of a vaginal formulation^{5,29}. Inconsistent drug absorption behavior may also be a concern due to the physiological variability observed during different stages of women development and hormonal status (e.g. childhood, pre- or post-menopausal, pregnancy).

Ideally, vaginal drug delivery systems should not interfere with vaginal physiology and daily life, while allowing obtaining high drug bioavailability (either local or systemic) with little variability and low incidence of side effects.

I.2. Vagina: anatomy, histology and physiology

The vagina is described as an expandable, longitudinally S-shaped, fibromuscular, collapsed canal showing at transverse cross-section an H configuration, with the anterior and posterior walls contacting each other in current conditions. It extends from the cervix of the uterus to the vestibule^{2,30,31}, presenting approximately 7-10 cm in length, more than 4 cm in width and 150-200 μm in thickness. The posterior wall is longer than the anterior, a consequence of the asymmetrical position of the cervix at the vaginal vault³²⁻³⁵.

Though it normally does not have secreting glands, the vagina is usually referred as a mucosa. In fact despite not having a secreting role, the vaginal epithelial surface is actually coated by a thin layer of fluid that includes endometrial, cervical and vestibular secretions, tissue transudate, residues of urine and products of cellular autolysis^{36,37}. Additionally, the composition of the vaginal fluid varies according to age, menstrual cycle phase and health status condition^{32,38}. Generally, it is considered that around 0.50-0.75 g of vaginal fluid are present in the vaginal cavity of a healthy reproductive aged women, representing a total daily production of 6 g³⁷. For instance, the vaginal pH is acidic (3.5-4.5) in healthy women during the reproductive age but it fluctuates along the different stages of the menstrual cycle and it is also dependent on coitus frequency, the amount of cervical mucus present in the vagina, the amount of vaginal transudate which also varies along the vagina (being higher close to the cervix and lower at the anterior fornix)^{2,37}. The maintenance of the pH is accomplished by lactic acid bacteria, mainly *Lactobacillus spp.*, microorganisms that metabolize into lactic acid the mono and di-saccharides that result from the autolytic breakage of vaginal cells glycogen³².

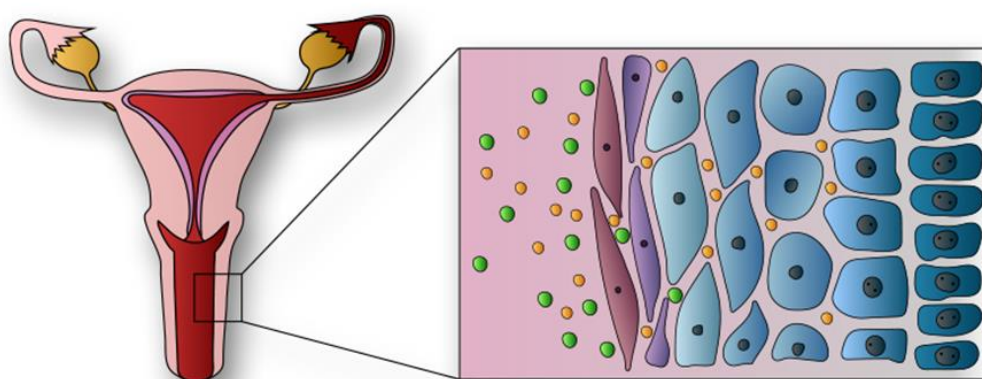


Figure I.1: Schematic representation of nonkeratinized stratified squamous vaginal tissue, also representing local and systemic drug delivery³⁹. Adapted from: Schnell, *Citología y Microbiología de la vagina*⁴⁰.

The vaginal wall consists of various cell layers: nonkeratinized stratified squamous epithelium, *lamina propria*, muscular layer and *tunica adventitia* covering only their proximal segments (**Figure I.1**). The *lamina propria* is constituted of connective tissue rich in blood and lymphatic vessels draining to the internal iliac vein, explaining why the absorbed products do avoid the hepatic circulation as an initial passage³¹. The vaginal cell turnover is estimated to replace 10-15 layers in a week⁴¹. The non-keratinized stratified squamous epithelium, settled on glycogen containing keratinocytes but also integrating other cell types (such as macrophages and Langerhans' cells), is grounded on the *lamina propria*, that includes elastic fibers, polymorphonuclear leukocytes and occasional lymph nodules⁴². The vaginal epithelial cells are disposed according to different stages of differentiation, identifiable through different keratins expression, such as K10 and K13, being the differential expression arrangement function of the cell location within the epithelium⁴³. Numerous folds and microridges called "*rugae*" are present in the epithelium, largely increasing the vagina's surface area and providing distensibility⁷.

The vaginal innervation depends on two types of sources: a peripheral one providing a highly sensible lower quarter segment and an autonomic fiber network in the upper tract, which is more sensitive to stretch than to touch or pain. This explains why women do not feel discomfort when using continuous intravaginal drug delivery systems⁷.

Several conditions influence vaginal physiology: hormonal balance, pregnancy, pH, microflora and age, being the last one the best current biomarker for epithelium layer thickness, enzyme concentrations and vaginal fluid production⁴⁴. Variation in these vaginal characteristics changes influence drug permeation as it depends mainly on the superficial layer characteristics, such as thickness, cell tightness, and lipids composition and organization in the intercellular space^{34,45,46}.

I.3. Vaginal dosage forms

I.3.1. Traditionally used in therapeutics

According to Portuguese Decree-Law no 176/2006 of 30th August, corresponding to the Medicines Statute, a pharmaceutical dosage form is defined as "the final state that active substances present after undergoing the necessary pharmaceutical operations in order to facilitate its administration and get the most desired therapeutic effect". Furthermore, the European Pharmacopoeia 8.0 states that vaginal preparations are liquid preparations, semisolid or solid intended to be administered vaginally, usually to a local action. They contain one or more active substances into an appropriate excipient. Containers intended for the packaging of vaginal preparations must meet the specified requirements. Various categories of vaginal preparations are defined: pessaries; vaginal tablets; vaginal capsules; vaginal solutions, emulsions and suspensions; tablets for vaginal solutions and suspensions; semisolid vaginal preparations; vaginal foams and medicated vaginal tampons⁴⁷.

In terms of production, it is stated that, during development, it must be demonstrated that the nominal contents can be withdrawn from the container of liquid and semisolid vaginal preparations presented in single-dose containers. In the manufacturing, packaging, storage and distribution of vaginal preparations, suitable measures are taken to ensure their microbial quality. Uniformity of dosage unit, uniformity of content, uniformity of mass and dissolution or disintegration profile constitute the specified testing for quality control of these dosage forms⁴⁷.

Generally, the most commonly administered dosage forms by the vaginal route are ovules, tablets and semisolids (in which category, creams). They are used as astringent drugs, antimicrobials (antibacterials, antiprotozoals, antifungals, antivirals), keratoplastics, as wound healers, spermicides, prostaglandins and steroids⁴⁸. However, these traditional commercial preparations have shown to have a short retention time due to the vaginal depuration mechanism, which results in discomfort and runoff feeling; are uncomfortable to apply; often require multiple daily administrations in order to obtain the desired therapeutic concentration and do not provide a uniform distribution of the drug^{5,49-51}. These characteristics generally entail a decrease in the acceptability of women in relation to these dosage forms, and so, leading to less reliability on the treatment.

I.3.2. Main evolutions on vaginal therapeutical strategies

The vaginal route is now considered an option for several therapeutic strategies. Hormones and antibiotics have been largely included in vaginal dosage forms but recently other therapeutic purposes have also been explored, such as prevention of infection and immunization. Also, the possibility of systemically delivering through the vaginal route, molecules presenting high molecular weight, such as calcitonin and insulin, has been explored^{52,53}.

The vaginal route is being increasingly used for hormone administration as it exhibits the great advantage of preventing gastrointestinal side effects and the hepatic first-pass effect. This is clearly useful for molecules that undergo a high degree of hepatic metabolism such as natural estrogens. While vaginal administration of estriol is indicated for atrophic vaginitis treatment in postmenopausal women, ethinylestradiol and etonogestrel are included in a vaginal ring for combined contraception. Vaginal progesterone is very effective in hormone replacement treatment (HRT) for postmenopausal women, in assisted reproductive technologies protocols and in supporting early and pre-term pregnancy^{54,55}. In pre-term labor prevention, indomethacin administered intravaginally has also been found useful and even advantageous when compared to its oral administration⁵⁶. The vaginal route is also considered the best option when the opposite aim is intended: labor induction. Vaginal misoprostol or dinoprostone are widely used for this purpose⁵⁷.

Over the last years, hormonal contraception has been achieved mainly by the oral or transdermal routes. However, the introduction of oral hormone pills into the vagina was shown to have relatively good efficacy and acceptability rates, opening ways for vaginal hormonal contraception^{58,59}. Nonetheless, it was the introduction of the vaginal ring which mainly promoted the vaginal route for hormonal contraception⁶⁰. Vaginal rings for steroids release have also been proposed when both local and systemic effects are intended, as is the case for estradiol to treat atrophic conditions including vaginitis^{61,62}. Moreover, distinct applications may emerge in the future for vaginal rings. For instance, it may contribute for the development of a new delivery approach for spermicides that are classically administered through semisolid dosage forms and sponges, and even microbicides. New spermicides molecules have also been developed and classic dosage forms have been proposed^{63,64}.

Regarding vaginal antimicrobial treatments, therapeutic strategies for the most common infections, namely bacterial vaginosis (BV) and vulvovaginal candidosis (VVC), include drug products for vaginal application. Clindamycin cream and metronidazole gel are two available therapeutic options to treat BV⁶⁵. However, due to increasing bacterial resistance and infection-related complications, acid-buffering gels and vitamin C tablets for vaginal application have been proposed, alone or combined with oral therapy⁶⁶⁻⁶⁸. Additionally, recognized antimicrobial molecules such as fenticonazole, garenoxacin and rifaximin have been re-visited and their possible topical application for BV treatment considered⁶⁹⁻⁷¹. Additionally, classical local therapies, namely with gentian violet solution and boric acid vaginal capsules, have been revised^{72,73}.

Vaginal administration of natural products to control and eradicate genital infections is very popular among women and arises as a possible alternative to overcome antibiotic resistance. Distinct plant extracts and essential oils have been proposed as valuable therapeutic alternatives for both BV and VVC, and have been studied *in vitro* and in animal models⁷⁴⁻⁷⁶.

Immunomodulation through the vaginal route is another important investigation field encouraging scientific work. Intravaginal administration of vaccines was shown to promote local immunoglobulin production, standing-up as a valuable route for sexually transmitted diseases

prevention^{77,78}. Also, a combined an anti-allergic and antifungal therapy based on oral cetirizine and fluconazole was tested and showed to be helpful in women suffering from recurrent VVC with persistent pruritus⁷⁹. Vaginal application of these two drugs may arise as a possible therapy.

Local therapy is also very common in human papillomavirus (HPV) lesions, as systemic therapy is highly ineffective⁸⁰. Podophyllin and podophyllotoxin, trichloroacetic acid solution and 5-fluorouracil have been used for vulvar infections. However, they must be very carefully used when applied in the vagina so that applications are restricted to diseased tissue⁸¹. Recently, new possibilities for the management of HPV infection have been proposed such as cidofovir, lopinavir, polyphenon E and an extract from green tea (*Camellia sinensis*)⁸²⁻⁸⁴. Immune response modifier molecules, such as immunostimulatory oligonucleotides and resiquimod, are also anticipated as a valuable vaginal topical therapeutic strategy to treat HVS genital infections and reduce the frequency of recurrences^{85,86}.

Topical administration of protective microorganisms, especially *Lactobacillus* spp., has been proposed to restore the vaginal microbiota after an insult and as an alternative or coadjuvant treatment for urogenital infections^{87,88}. Clinical trials have shown vaginal probiotics formulations to be safe and having high rates of acceptability but data on efficacy of these formulations are still controversial, mostly related to limitations such as small samples studies and lack of product stability^{89,90}.

I.3.3. Less used/advanced dosage forms

The major challenge in vaginal dosage forms design is the ability to fulfill functional criteria such as product dispersion throughout the vagina, prolonged residence time, adequate physicochemical interaction with vaginal content, release profile of active ingredients and effects on targets⁹¹.

Bioadhesion has been stated as a target characteristic for the design of new vaginal products since it is defined as the attachment of synthetic and natural macromolecules to a biological tissue and mucoadhesion is considered a particular case of bioadhesion when the biological tissue is covered by mucus⁹². Mucoadhesive polymers have been explored in the development of either semisolid and solid vaginal drug delivery systems in order to circumvent some of the outlined limitations of traditional dosage forms while gathering user compliance and improving therapeutic outcomes⁹³. Moreover some polymers have shown microbicidal activity on their own, as is the case for kappa carrageenan (absorption inhibitor, *in vitro*), carbomers (interruption of HIV cell binding, *in vivo*), cellulose acetate phthalate (inactivation of HIV and HSV, *in vivo*), and polystyrene sulfonate (activity against HIV and HSV, *in vitro*)¹.

Herein this study, and regarding the pharmacopeia background, tablets, pessaries, capsules, gels, creams and solutions are considered as the traditional dosage forms for vaginal administration²⁶. In addition, over the last decades, new vaginal drug delivery systems (such as rings, films, sponges and diaphragms or cervical caps) have gained popularity among pharmaceutical developers, clinicians and users, and are now being the focus of intense study.

Vaginal rings are doughnut-shaped polymeric dosage forms that have been initially developed back in the 1970s for the delivery of hormones for contraceptive purposes⁹⁴. Vaginal rings have adequate flexibility and dimensions in order to allow comfortable insertion and retention in the vagina. Different cross-sectional configurations have been proposed presenting diverse properties, particularly related with their ability to provide controlled drug release, and requiring different manufacture processing²⁶. One of the main advantages of vaginal rings is the possibility to release one or more drugs in a sustained fashion for up to one year, abbreviating problems such as compliance or daily fluctuation of drug levels, as in the case of oral contraceptives. Also, rings retain their shape throughout the time of application and can be removed in case of need⁹⁵. In present days, one vaginal ring releasing a combination of hormones (etonogestrel/ethinyl estradiol) is commercially available worldwide for contraception purposes (Nuvaring®/Circlet®, Organon currently Merck Sharp & Dohme) while two others containing estradiol (base or acetate) are used for hormonal replacement therapy in post-menopausal women (Estring®, Pfizer and Femring®, Warner Chilcott). Currently, vaginal rings are being developed for the delivery of antiretroviral drugs to be used as microbicides and non-hormonal contraceptives^{96,97}.

The progress in polymer science has provided thin films with the flexibility to meet the demands and requirements for use as drug delivery systems⁴⁹. Vaginal films are polymeric drug delivery systems usually square in shape, with soft and homogeneous surfaces that can be applied without the use of an applicator⁹⁸. These dosage forms are thin strips of polymeric water-soluble substances which disperse/dissolve when placed in the vaginal cavity to release the active pharmaceutical ingredients⁹⁹. Films are designed to rapidly disperse or dissolve in contact with fluids. These desirable features are expected to facilitate insertion, gather user's acceptability and compliance, and result in the immediate dispersion with formation of a bioadhesive vaginal coating that could be retained in the vagina for prolonged periods of time^{91,100}. Vaginal films have mostly been approved as spermicides, although they present inferior contraceptive success than hormonal methods, condoms and intrauterine devices. Several vaginal film formulations are under investigation with diverse therapeutic goals being contraceptives and microbicides the most explored examples among them^{10,50,51}. Vaginal sponges, are formulated similarly to vaginal films. Usually they are impregnated with one or more drugs in order to be inserted in the vagina for a limited amount of time, usually up to 24 h. Their application is essentially as non-hormonal contraceptives but other uses have also been proposed (e.g. in the treatment of bacterial vaginitis). One commercially available example is the Today® sponge (Almatica Pharma, Inc.) which comprises a soft polyurethane sponge (7.6×3.8 cm) impregnated with one gram of the spermicide nonoxynol-9^{63,101}.

Other types of vaginal barrier devices, namely diaphragms or cervical caps, have also been modified in order to deliver spermicides or microbicides^{102,103}. Drugs may be impregnated in the matrix of the device, or applied into a specific reservoir or simply placed on top of the device prior to insertion. Other authors proposed dosage forms including vaginal patches similar to those used on the skin but with adequate size for treating small mucosal areas, namely localized

cervical neoplastic lesions¹⁰⁵. Finally, vaginal inserts containing dinoprostone have been long used in the clinics for cervical ripening or labor induction (Propess®, Ferring, and Cervidil®, Forest Laboratories)¹⁰⁶.

The relatively low accessibility to the vaginal canal may justify the use of applicators in order to correctly administer a dosage form. While in the case of foams, semisolids and liquids this is mandatory (namely when deep insertion is required), for solid systems their use may be optional or even unnecessary. Special applicator tips can also be attached to a tube containing a liquid, semisolid or foam in order to be inserted in the vagina. In all cases, applicators (or applicator tips) may present different designs according to specific needs or women's preferences and so they have been also the subject of studies and innovation¹⁰⁴. In general, these medical devices should be made of non-toxic materials (e.g. polypropylene, polyethylene), allow comfortable administration to the desired site within the vagina (e.g. placement near the cervix, allow optimal distribution throughout the mucosa), and avoid any damage to the mucosa. Also, affordability may be an important question particularly when considering products intended to be used in low-resource settings.

1.3.4. “*Mind the gap!*” Marketed products vs R&D products

Different interesting new approaches have been proposed over the last years in order to improve vaginal drug delivery. New drug delivery systems have been researched to respond to the challenges posed by the vaginal anatomy and physiology. One interesting strategy has been the development of stimuli-sensitive systems, which can modify their behavior (e.g. drug release rate, physical state) once the environmental conditions change. A straightforward approach is to take advantage of the increase in temperature upon vaginal administration. Different thermosensitive formulations based on poloxamers have been proposed for vaginal drug delivery. These systems are liquid at room temperature but become gels at near body temperature. This allows for easy administration and intravaginal distribution typical of a liquid but with enhanced retention due to gelation. Further, thermosensitive systems usually comprise mucoadhesive polymers which strengthen their ability to reside intravaginally¹⁰⁷⁻¹⁰⁹.

Modified drug release is also typically observed for this type of systems. Alongside temperature, systems that can respond to changes in vaginal pH can be an interesting mean to modulate drug release. For instance, Gupta *et al.* developed a thermo- and pH-sensitive system based on a terpolymer of *N*-isopropyl acrylamide, acrylic acid, and butyl methacrylate which allows adequate administration, distribution and retention due to its thermosensitive nature¹¹⁰.

The use of microparticles has been proposed for drugs intended to be administered by the vaginal route. Main advocated advantages for these systems are the possibility of obtaining control over drug release, provide enhanced retention by using mucoadhesive polymers and protect the drug payload^{51,111,112}. Typical processing of developed microparticles into tablets or gels has been proposed in order to allow administration. Further, larger starch-based particles (pellets) have been proposed by Vervaet and collaborators, which showed to provide an interesting approach for complete distribution and prolonged retention of drugs intravaginally,

as assessed in both sheep and humans^{113,114}. Nanotechnology-based solutions for improving vaginal drug delivery have been increasingly proposed over the last years, particularly to be used in microbicide development¹¹⁵. Developed systems include liposomes and proliposomes, niosomes, polymeric nanoparticles, and solid lipid nanoparticles¹¹⁶⁻¹²². Claimed advantages of nanosystems include the ability to allow protecting sensible drug payloads (e.g. peptides, proteins and genetic material), improving solubility, obtaining controlled/sustained drug release, and achieving mucosal penetration and targeting special cell types (e.g. HIV-target cells)¹¹⁵.

Alongside the use of probiotics for prevention and therapeutic purposes, the vaginal microbiota provides interesting opportunities to vaginal drug delivery. Indeed, engineered commensal bacteria may provide an interesting live “platform” for the vaginal delivery of active substances. In particular, bacteria producing antiviral compounds have been tested in order to prevent the sexual transmission of HIV and for vaccine development¹²³⁻¹²⁵.

Although these formulation strategies may represent great advances in the field of vaginal products, their translation into the market is not yet fully achieved. The incorporation of these strategies into traditional or modern dosage forms may provide women with vaginal products characterized by improved performance regarding efficacy and safety. However, it shall not be forgotten that effectiveness is highly dependent on therapeutic adherence. So, women’s preferences shall be considered along the product development process.

I.4. Women's acceptability for vaginal products

Acceptability of a vaginal product represents a major factor for effectiveness since it clearly influences correct and consistent use, especially when long-term use is required. Most available data on this topic have been obtained from clinical trials, experiences with the use of surrogates (in the case of microbicides) and interviews assessing women's attitudes upon product demonstration (particularly willingness to use or to recommend the product), mainly in the context of contraceptives and microbicides, where small samples are frequently evaluated¹²⁶⁻¹²⁹.

In clinical practice, however, the major use of vaginal products is related to vaginal infections, which represent the most frequent reason for women to seek medical consultation¹³⁰. Several surveys have estimated that more than 70% of adult women have had a vaginal problem and have used vaginal products to treat infections^{23,131,132}.

The use of traditional vaginal dosage forms has been associated with insertion difficulties and low residence time resulting in leakage and discomfort. Improvement of vaginal dosage forms has been achieved by either developing formulations with increased residence time (mainly by using bioadhesive polymers) or new delivery systems such as rings and films^{14,26,93}.

The development of more appropriate vaginal products must, obviously, consider women's preferences, and these may, in turn, depend on their age, socio-economic status and cultural backgrounds¹³³ and on the type of product they would need to use. For products used for contraception or to treat vaginal infections, opinions collected from reproductive aged women in particular shall be searched, while for hormonal replacement therapy the opinion of older women shall be more adequate. Also, women's fears and misconceptions on the safety of these products shall be addressed by prescribers and educational strategies shall be adopted to assure compliance and therapeutic success. Additionally, misconceptions and fears related with the use of vaginal products are assessed and results are compared with data available from previous studies conducted across other populations¹³¹.

A large scale online survey was conducted to assess Portuguese women's experiences, preferences and general perspectives on the vaginal route for drug delivery and on vaginal products. Women's preferences on the physical properties and mode of insertion of vaginal products were analysed according to age groups. Women preferred vaginal products to be odourless and colourless gels, creams and ointments composed by drugs/excipients of natural origin and applied by means of an applicator. Although, the majority of women would prefer not to insert any product in the vagina, intention to use for themselves and recommendation to use for others was associated with previous experiences with vaginal products. In this population, general concerns and misconceptions related to use of vaginal products were rare. Overall, semisolids were the most used and preferred vaginal products, while vaginal rings were highly acceptable for women who had previously used them. Although they considered the vaginal route to be more efficient and safe, many women felt it to be less appealing than the oral route, particularly due to comfort issues.

Semisolid formulations are the ones preferred by women although they might conduct to some extent of discomfort if not properly formulated. These systems are easy to use, have good acceptability and provide relatively inexpensive options for drug therapy. However, leakage, messiness and discomfort during application are recognized as important limitations. In the particular case of leakage, night administration is usually recommended. Vaginal creams present the possibility of easily dissolve both hydrophobic and hydrophilic drugs even in the same formulation. Their main application has been focused on the delivery of hormones and antimicrobials^{134,135}. In the case of vaginal gels, main advocated advantages are their ability to provide high bioavailability, biocompatibility and spreadability¹³⁶. Also, the use of polymeric gelling agents usually provides mucoadhesive properties to these dosage forms which can increase vaginal retention and reduce leakage. Mostly hydrophilic in nature, vaginal gels are generally easy to use, inexpensive and highly accepted by women, being usually associated with a refreshing effect due to its high-water content. Aqueous and non-aqueous vaginal gels have been the main dosage form used for developing microbicides^{137,138}. One common challenging problem in drug formulation is the low solubility of most active substances. In the particular case of the vaginal route, the limited amount of fluid present in the vagina and relatively low amount/size of a product that can be administered intra-vaginally may enhance solubility issues. In order to obviate such problems, formulations comprising the use of cyclodextrins¹³⁹, microparticles¹⁴⁰ or multiple emulsions¹⁴¹ have been successfully used. However, a great deal of work is still required in the field.

This type of formulation is already largely commercialized for vaginal administration, and simultaneously, scientific research is introducing new excipients and techniques to its early stages of development, production and characterization.

I.5. Early safety assessment

Ideally, vaginal drug delivery systems should not interfere with vaginal physiology and women's daily life, while allowing obtaining high drug bioavailability with little variability and low incidence of side effects¹⁷⁻²¹.

Over the last decades, efforts have been made to conduct investigation to a level of high comprehension of vaginal epithelium mechanisms⁶. The understanding and application of several techniques to predict drug toxicity to and through the vaginal epithelium contributes to the successful selection of drugs and appropriate formulations in the early stages of development⁴². Thus, it is of great importance to implement accurate and reproducible methods to predict drug toxicity. The *in vitro* and *ex vivo* approaches should be privileged, and preferably an optimal *in vitro/in vivo* correlation should be established. Some of these methods and techniques are widely available and they should be used to address biocompatibility and irritation potential in early stages of product development¹⁴². The strategy of patient-driven product development is also currently reflected in policies such as the biopharmaceutics risk assessment roadmap (BioRAM) which aims to optimize drug product development and performance by using therapy-driven target drug delivery profiles as a framework to achieve the desired therapeutic outcome. Simultaneously, clinical relevance is directly correlated to early formulation development^{143,144}. Biopharmaceutic tools are used to identify and address potential challenges to optimize the drug product for patient benefit. Recently, a Scoring Grid was developed to facilitate optimization of clinical performance of drug products (easier addressing of critical questions and decision making)¹⁴⁵.

In the field of vaginal administration, preclinical development published innovation has been largely devoted to microbicide formulations. However, the same safety/risk assessments could be transposed to other vaginal product categories. In fact, preclinical development, i.e. nonclinical access to toxicological and pharmacokinetics data, is not only determinant and prospective for clinical development, but also, could be optimized in order to reduce general financial costs and overall time of product development before commercialization¹⁴⁶⁻¹⁵⁰.

I.5.1. Technological characterization

One of the serious problems associated with the formulation and manufacture of topical-mucosal preparations is the establishment of reliable techniques for their characterization, mainly because of the complexity of their physical structure¹⁵¹. Ultimate acceptability and clinical efficacy of such preparations require them to possess optimal mechanical properties (ease of removal from the container, spreadability on the substrate), rheological properties (viscosity, elasticity, thixotropy, flowability), and other desired properties such as bioadhesion, desired drug release, and absorption¹⁵².

Some of the technological characteristics of vaginal products are also directly related with their safety when applied as is the case for pH and osmolality. Hyperosmolar vaginal formulations have already been associated with mucosal damage and local side effects^{153,154}.

Moreover, formulations capable of lowering the normal vaginal pH may cause vaginal mucosa damage, while alkaline products may potentially lead to decreased levels of protective *Lactobacilli*^{155,156}. Of course, achieving all such properties remains challenging. Several formulations have been developed or are currently under development satisfying these recommendations^{157,158}. Since these two physical characteristics can be determined just upon formulations preparation by using quick and easy-to-use methodologies, they represent a double advantage for product characterization thus predicting irritation potential. Alongside, several vaginal lubricants have already been characterized in terms of pH, acid-buffering capacity, osmolality and cytotoxicity in order to check for vaginal epithelial irritation prediction, since the World Health Organization (WHO) published an advisory note stating that osmolality over 1200mOsmol/kg could denote potential irritation¹⁵⁹.

I.5.1.1. pH and acid-buffering capacity

It is well established that deviations from the normal vaginal pH in the healthy adult (3.5-4.5) are considered as potentially deleterious for the vaginal epithelium¹⁶⁰. The acidic pH in the vaginal environment contributes to its normal physiology, microbiota, and a balanced immune response. Vaginal products should be compatible with vaginal pH and, ideally, maintain it or even help in its reestablishment (e.g., in cases of bacterial vaginitis or menopausal women)¹⁶¹. Even if the consequences of the administration of vaginal formulations presenting undesirable pH is not readily assessable, it is well known that increased vaginal pH is associated with the presence or favouring of bacterial vaginosis, trichomonosis or mixed infections¹⁶². Outcomes of low pH are even less understood, but animal data suggest that values of three or less are unacceptable for human use¹⁵⁵. Furthermore, apart from being pH compatible, vaginal products should allow the maintenance of the vaginal acidic environment and oppose pH-raising/decreasing events. Indeed, the use of acid-buffering gels has been proposed for the reestablishment of pH in cases of infection^{66,157} or menopausal atrophy¹⁶³.

A study of pH and acid-buffering capacity of diverse marketed vaginal lubricants has already been performed within our workgroup¹⁵⁹ and it concluded that most of the lubricants presented pH and/or osmolality values outside the ranges recommended by the WHO. With this study it was made clear the need for further characterization in order to fully understand the potential hazard profile of the vaginal products.

I.5.1.2. Osmolality

Vaginal products' safety goes beyond chemical toxicity to include physical parameters such as osmolality¹⁶⁴. If a formulation is excessively hyperosmotic there is the potential to cause irritation leading to an inflammatory response. Each dissolved ingredient in a topical formulation contributes to the final product osmolality, so the effect of the overall composition of the formulation must be considered in addition to the impact of each individual ingredient. In general, the loss of cell viability is possibly correlated to the gels' osmolality; the higher the solute concentration, the greater the dilution that is needed to maintain viability^{159,164}.

The World Health Organization (WHO), in collaboration with the United Nations Population Fund (UNFPA) and Family Health International (FHI360), recently issued an “Advisory Note” on the technical requirements of vaginal lubricants, namely when used in addition to condoms. Osmolality has been highlighted, and specific recommendations have been proposed: values of 380mOsmol/kg or lower are desirable (hypo- and isosmolal), but values as high as 1200mOsmol/kg have been considered acceptable on an interim basis¹⁶⁵. Available pre-clinical and clinical data support that hyperosmolal vaginal products may be related to safety issues¹⁶⁶, as well as detrimental effects on sperm motility, viability and chromatin^{167,168}.

I.5.1.3. Texture

Textural analysis is performed in semisolid formulations to understand the mechanical characteristics of the formulation^{169,170}. One of the tests that can be performed to access textural properties is the penetration test, where an analytical probe is depressed into the sample at a defined rate to a desired depth. From the resultant force-distance curve, the mechanical parameters of firmness and adhesiveness may be derived. Firmness is defined as the force necessary to attain a given deformation and adhesiveness is defined as the work necessary to overcome the attractive forces between the surfaces of the sample and the surface of the probe with which the sample comes into contact¹⁷¹.

I.5.1.4. Bioadhesion

Mucoadhesion, herein referred as bioadhesion since the vaginal epithelium is not considered to be a mucosa¹⁷². Bioadhesive properties allow better contact of the formulation with the vaginal surface and longer residence times. The mechanisms of bioadhesion involve firstly a contact stage, where hydration, wetting and spreading are the most important steps, and subsequently a consolidation stage, that involves the strengthening of polymer-mucin joint, thanks to the inter-penetration of the polymer chains into the mucus layer and the occurrence of polymer-mucin bonding (mainly weak van der Waals and hydrogen bonds or electrostatic interactions)^{172,173}. Maximum force of detachment (F_{\max}) (directly measured) and the work of adhesion (W_{ad}) (calculated as the area under the force vs displacement curve) are the parameters used to evaluate the bioadhesive potential. The reliability of tensile method is strictly dependent on the failure in the interfacial (mucin/polymer) region: in particular it is difficult to distinguish where the failure of the bioadhesive joint occurs and whether the cohesive nature of the sample (failure within the polymer layer) or the strengthening of the mucus layer (failure within the mucus layer) plays the major role¹⁷³.

The natural mild slope of the vaginal canal, in association with its self-cleansing mechanisms (e.g. fluid secretion) and possible mechanical stress (e.g. during penile penetration), contributes to the expulsion of products placed in the vagina. Another important issue impacting the mucoadhesion phenomenon is related to the variability of the vaginal fluid during the menstrual cycle, vaginal practices (e.g. douching) or sexual intercourse. Vaginal fluid can undergo either quantitative or qualitative changes, namely in pH, mucin content and

rheology. These factors influence the interaction of mucoadhesiveness with mucin, namely by changing the conformation and properties of the network formed by mucin within the vaginal fluid¹⁷⁴.

Mucoadhesive dosage forms or delivery systems can contribute to prolonged *in situ* residence, resulting in advantageous features such as fewer applications needed, reduced vaginal leakage, and intimate contact between drugs and the mucosal tissue. Different dosage forms discussed above have been formulated as mucoadhesive, namely tablets, suppositories, creams, and gels⁹⁸. Indeed, one of the first enthusiastic reports on a specific mucoadhesive vaginal gel dates back to the 1990s by Robinson and Bologna¹⁷⁵. The mucoadhesive properties of the proposed gel, currently commercialized as Replens® (Lil' Drug Store Products, Inc.), were attributed to the inclusion of an acrylate polymer, polycarbophil (1-3%). Since then, these polymers have been used as classical mucoadhesive and gelling agents for the formulation of various commercially available vaginal gels¹³⁶. More recently, Garg *et al.* proposed a new mucoadhesive gel, ACIDFORM which was shown to present enhanced *in vitro* mucoadhesive properties when compared to various commercial gels¹⁵⁷.

The common strategy for increasing mucoadhesiveness of vaginal dosage forms has been to use well known mucoadhesive polymers such as polyacrylates, chitosan, cellulose derivatives, hyaluronic acid and derivatives, pectin, starch, and several natural gums, among other⁹³. Acidic polymers such as polyacrylates present the additional feature of allowing buffering the vaginal pH at its desirable normal values, and thus potentially contribute to a healthy vagina¹⁷⁶. As for chitosan, its intrinsic ability to interact with intercellular tight junctions and inhibit proteolytic enzymes provides additional mechanisms to promote the vaginal absorption and peptide/protein protection from degradation, respectively¹⁷⁷. In recent years, thiolated polymers have also been tested for designing vaginal dosage forms and with improved mucoadhesive performance when compared to their non-thiolated counterparts¹⁷⁸. Even if substantial success has been achieved, much of the rationale behind the choice of mucoadhesive polymers for vaginal formulation derives from studies intended to evaluate these excipients for use in other mucosal routes¹⁷⁹. The mucoadhesive potential of polymers and derived dosage forms is also dependent on the specificities of the mucosal environment and its evaluation should take this into account. For instance, *in vitro* experimental settings relevant to the vaginal physiology, namely pH values, have been shown to significantly influence the mucoadhesive performance of vaginal semisolid formulations¹⁸⁰. This need for mimicking the vaginal environment led to the development of different specific *in vitro/ex vivo* experimental protocols for evaluating the mucoadhesive potential of vaginal dosage forms. Proposed techniques generally involve measuring the forces involved in the detachment of a formulation from a synthetic model or natural mucosa. Alongside, imaging techniques have been used to evaluate mucoadhesiveness *in vivo*²⁰.

I.5.1.5. Viscosity

The evaluation of rheological properties for the gel type dosage forms would be important for predicting their behaviour *in vivo*. The flow properties of semisolid vaginal dosage forms might be used to predict the spreading and coating of the formulations over the vaginal epithelia¹⁸¹. Viscosity could dictate the residence time in the genitourinary tract of these formulations hence it is decreased, owing to the self-cleansing action of the vagina and the dilution with vaginal fluids, and even environmental temperature¹⁸². Viscosity should be characterized not only directly on the formulations but also considering the dilution on the vaginal fluids and the vaginal temperature^{157,182}.

I.5.2. Alternative non-animal methods for safety characterization

Predicting product toxicity on the vaginal epithelium contributes to earlier and successful selection of drugs and appropriate formulations in the early stages of development⁴². Thus, it is of great importance to implement accurate and reproducible methods to predict drug toxicity. The *in vitro* and *ex vivo* approaches should be privileged, and preferably an optimal *in vitro/in vivo* correlation should be established. Furthermore, these strategies would rather be more useful in early stages of product development, by addressing preliminary biocompatibility and irritation potential¹⁴². Still, the standard method for assessing vaginal mucosal irritation is the *in vivo* rabbit vaginal irritation test, to what concerns medical devices and drug products^{42,183}.

The cosmetic industry under the supervision of regulatory agencies (FDA, ECVAM, OECD) has already been evaluating and adopting alternative *in vitro* methods for eye irritation, phototoxicity, skin irritation, sensitisation and corrosion¹⁸⁴. However, presently cosmetic and pharma industries are still not focused on validating standardised tissue models or cellular assays for vaginal irritation assessment, since this particular application represents yet a small part of business.

Preclinical safety non-animal testing includes the assessment of toxicity in cell- and tissue-based assays (including assays with epithelial cell lines, peripheral blood mononuclear cells, primary epithelial cells, and cervical explants). Furthermore, while the toxicity profiles of candidate drugs are generally assessed on *in vitro* screening assays (cell-based assays, tissue explants), this is not the case for excipients. Selection of excipients is mainly based on their functionality and, if available, on data resulted from previous use in vaginal formulations, as can be found in the FDA list of inactive ingredients¹⁸⁵.

Therefore, as long as there is no safety certainty before heading to *in vivo* experimentation, potential safety issues detected in *in vitro/ex vivo* assays should be seriously considered. The international efforts to identify vaginal biomarkers, including cytokines and chemokines, are the way forward to correlate *in vitro* and *in vivo* safety testing more properly¹⁸⁶⁻¹⁸⁹, along with validated methods that could predict vaginal toxicity. Ideally, a drug/formulation candidate should not damage or interfere with the epithelial integrity and

function. To foresee this, rigorous preclinical testing with various *in vitro* assays covering cell toxicity, along with the evaluation of changes in epithelial integrity and immunological functioning of epithelial cells should be required¹⁹⁰.

1.5.2.1. *In vitro* cell-based assays

Cell-based assays are generally used to quickly screen collections of compounds in order to determine if they have effects on cell proliferation or show direct cytotoxic effects that lead to cell death¹⁹¹. Cell-based assays also are widely used for measuring receptor binding and a variety of signal transduction events that may involve the expression of genetic reporters, trafficking of cellular components, or monitoring organelle function. Actually, a wide variety of methods able to estimate the number of viable eukaryotic cells are available. This procedure is usually conducted on a multi-well format and data is easily recorded using a plate reader (by spectrophotometry or fluorimetry). As a general picture, the methods mainly applied include: reduction/uptake assays, protease markers, and ATP detection¹⁹². The reduction/uptake, and protease activity assays evaluate the general metabolism or an enzymatic activity as a marker of viable cells. All of these assays require incubation of a reagent with a population of viable cells to convert a substrate to a coloured or fluorescent product that can be detected with a plate reader. Under most standard culture conditions, incubation of the substrate with viable cells will result in generating a signal that is proportional to the number of viable cells present. When cells die, they rapidly lose the ability to convert the substrate to product. That difference provides the basis for many of the commonly used cell viability assays. The ATP detection assay is different because the addition of the selected reagent immediately ruptures the cells, thus there is no need of further incubation period with the testing substances.

Cytotoxicity assays constitute a gold standard at *in vitro* preclinical evaluations of chemicals in cultured cells. The MTT reduction assay, included in the tetrazolium reduction group, is one of the most common applied. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a water soluble yellow tetrazolium salt, which is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria. This product is impermeable to cell membranes, and consequently it accumulates in metabolic viable cells¹⁹¹. Another commonly applied cytotoxicity method is the Neutral Red Uptake (NRU) assay. It is also a quantitative colorimetric method, based on the ability of viable cells to incorporate and bind (uptake) the red dye within the lysosomes^{193,194}. Although used extensively as convenient and rapid measures of cell viability, all these methods should be carried out with some caution. As a general remark, these assays have in common with other cell culture procedures, limitations concerning the test substance characteristics (volatile, unstable or explosive in water, low solubility)¹⁹⁵.

Actually, this kind of assays are already approved in normatives such as ISO (10993-5), which standardizes *in vitro* evaluation of medical devices, including the ones for vaginal application, recurring to rodent cell-lines testing¹⁹⁶.

I.5.2.2. *Ex vivo* tissue-based assays

Tissue culture represents higher structure and response complexities when compared with cellular cultures. Furthermore, it has been stated that tissue fidelity and function are best achieved when using tissue biopsies or explants *ex vivo*, rather than reconstructed tissues from individual cell types¹⁹⁷. This means that actually, *ex vivo* models can have an important role in toxicity testing, alongside with cellular models, before proceeding to *in vivo* animal models. Porcine vaginal explants have already been applied to the toxicity determination of antiseptics¹⁹, and are proven to have a high level of similitude with the human vaginal epithelia (Figure I. 2). This surrogate is easy to obtain, is economic, has no ethical limitations and is more suitable to test final formulations (due to its robustness) so it has a high potential to be addressed as an innovative preclinical tool to study not only toxicity profiles, but also, drug permeation (as described on the upcoming topics).

I.5.2.3. *Ex vivo* organ-based assays

To date, no organ-based toxicity assay has been proposed to assess toxicity induced through the vaginal administration route. However, the parallelism between sensitivity to eye and vaginal irritants has been described in the literature. Thus, if the level of sensitivity of the two organs is similar, possibly the techniques and methods for their irritation potential testing might be the same - ISO 10993¹⁹⁶. Eye irritation has some organotypic *in vitro* assays already validated or ongoing validation, such as the Bovine Corneal Opacity & Permeability test (BCOP), the Isolated Chicken Eye test (ICE), the Isolated Rabbit Eye test (IRE) and, the Hens Egg Test on the Chorio-Allantoic Membrane (HET-CAM). The HET-CAM assay is already widely employed for ocular products, yet its application for the testing of vaginal formulations is innovative, and was developed in the context of this thesis (see Chapter III). Briefly, the HET-CAM assay consists on evaluating outcomes (haemorrhage, lysis and coagulation) which are then calculated to give a final Irritation Score (IS) that attributes an irritation potential to the test product¹⁹⁸. The fact that this assay embraces the use of a complete organ further increases the complexity in structure, when compared to *ex vivo* tissue models and cellular-based models. Although using incubated Hen's eggs for tests could raise the question of *in vivo* testing, it does not conflict with animal protection laws. It was already demonstrated that incubation up to day nine, the embryonic differentiation of the chicken central nervous system is sufficiently incomplete to avoid suffering and pain perception. Actually, the few sensory fibers present at day nine only develop after incubation during 11 to 14 days¹⁹⁹. Studies also suggested that the extraembryonal vascular systems (e.g., yolk sac, CAM) are not sensitive to pain²⁰⁰.

I.5.3. Drug performance: from *in vitro* release to *ex vivo* permeation

The understanding and application of several techniques to predict drug permeation through the vaginal barrier contributes to the successful selection of drugs and appropriate formulations in the early stages of development. Thus, it is of great importance to implement

accurate and reproducible methods to predict drug permeation. Tissues explants (*ex vivo*), being more representative in structure and presenting more viability, shall be preferred over *in vitro* cellular methodologies. Standardization and validation of methodologies to be used by different research groups will be obviously valuable for uniform interpretation and extrapolation of test results. Moreover, these tests may be particularly important for the development and characterization of new products intended to reach the market since they provide vital information for marketing authorization purposes.

I.5.3.1. Drug absorption from the vagina

Drug dissolution in the vaginal fluid and epithelial penetration are the two key steps for a drug to be absorbed through the vagina. As a result all factors associated with vaginal physiology and formulation profile will greatly influence the success of drug delivery to the target^{6,7}. Even though the vagina is not a real mucosa, drug transport is accomplished in a multi-way mechanism similar to the other biological membranes. Drug absorption can occur passively or actively. Passive mechanisms include the transcellular route, through the cells' membrane, and the paracellular route, representing a diffusion process through intercellular fluid and tight junctions^{201,202}. Tight junctions and other intracellular junctions (adherens junctions and desmosomes) are present in the vaginal and cervical epithelium having a higher expression in the endocervix. Although the uppermost layers of the ectocervical and vaginal epithelia are devoid of tight junctions, these and other intercellular junctions have been identified right beneath the most apical epithelium layers. The study of these junctions is particularly important to understand, for example, the invasion of microbes and drug permeation. Tight junctions are composed of transmembrane proteins (occludin, claudins and junctional adhesion molecules - JAMs) which contact across the intercellular space and create a seal to restrict paracellular diffusion of molecules across the epithelial sheet. Furthermore, tight junctions have a structural role in epithelial polarization by limiting the mobility of membrane-bound molecules between the apical and basolateral domains of the plasma membrane of each epithelial cell. In general, tight junctions are responsible for sealing the epithelial barrier as well as the selective passage of small ions and fluid²⁰¹. The active transporters generate gradients across the barriers, being the mechanisms either a primary transcellular process or a secondary one. While the latter is indirectly coupled to adenosine triphosphate (ATP) energy, the primary active transport directly utilizes ATP during the transport cycle²⁰³. Nevertheless, previous studies on vaginal permeation of drugs show that most of the active substances permeate the vagina through diffusion mechanisms^{17,98}. Lipophilic substances, such as steroids, are mainly absorbed by the transcellular route³², in contrast to hydrophilic drugs which follow the paracellular diffusion mechanism¹⁷. Low molecular weight lipophilic drugs are more likely to be better absorbed than larger molecules or even hydrophilic drugs, independently of their molecular weight⁵.

Among several factors that do affect drug absorption from the vagina, both vaginal fluid amount and composition interfere in drug dissolution before transport. While an excessive fluid

content promotes a “washing” effect that decreases drug retention, cervical mucus presence is able to increase bioadhesion³². Estrogens, due to epithelial cell proliferation stimulation, influence drugs pharmacokinetics through the vagina, as a consequence of increased blood irrigation, transudation and cervical mucus production. Consequently, when a systemic action of a drug is desirable, several factors shall be taken into account, like the epithelium layer thickness, the enzymatic and hormonal cyclic changes, the vaginal pH (that interferes with ionization of electrolytic drugs) and sexual stimulation and intercourse^{204,205}.

Additionally, vaginal administration of a substance, being either a drug or an excipient, may cause irritation, inflammation and damage, leading to barrier disruption and so enhancing not only its absorption but also the penetration of infectious agents^{34,186}. Drugs and excipients’ physicochemical characteristics are also relevant for determining their absorption^{177,206}. The excipients present in the formulation have also an important role both in drug release and absorption profiles⁷. Molecules’ lipophilicity, ionization, molecular weight, surface charge and chemical nature represent the most relevant properties that determine their ability to permeate both the apical and basolateral membranes of the epithelial cells^{207,208}.

1.5.3.2. Vaginal drug permeation methodologies

The ethical and practical constraints to the use of human beings and their organs to assess drugs and formulations’ efficacy and safety, led researchers to create and develop alternative methods and models to predict drug permeation through the vagina. These include cells, tissues (both organs and culture systems) and full animals^{34,188,209-211}. Technical complexity, economic expenditures and ethical issues determine if a model will either be valid, applicable and reproducible or if it is not trustworthy to be used in laboratory research. **Table I. 1** gathers some of the main advantages and disadvantages of models to be applied for predicting vaginal drug permeation.

Table I. 1: Advantages and disadvantages of models applied for predicting vaginal drug permeation. Adapted from Costin *et al.* (2011)⁴².

	<i>in vitro</i>	<i>ex vivo</i>	<i>in vivo</i>
	Cell-based Reconstructed tissue	Tissue explants	Laboratory animals
Advantages	Cultures are relatively inexpensive and easy to grow.	Full cell structure (epithelial, connective, immune). Better tolerance to formulations. Parallel efficacy testing.	Complete organism features addressed: structurally, functionally and physiologically.
Disadvantages	No barrier function and no systemic component in pure cell cultures. Organotypic cultures tend to be more permeable than <i>in vivo</i> and have no vascular component.	Limited number. Institutional review board approval required. More technically demanding. Variability.	Time and cost expensive. Data still difficult to correlate to humans. Regulatory and ethical issues.

1.5.3.2.1. *In vitro* models

Over the last decades, cell-based *in vitro* models for drug permeation prediction allowed the use of simple, economic, reproducible and ethically accepted methodologies in the earlier stages of drug development. These models use both immortalized cells and primary cell cultures that are able to proliferate in monolayers, simulating the intended epithelium, both structurally and functionally²⁰⁸. Cell culture models can be easily manipulated, in terms of culture conditions and parameters, and represent good drug absorption predictors. Human cell lines or primary cell cultures circumvent problems related to the use of animal tissue on *in vitro* models²¹².

The main limitation of *in vitro* models, result from the impossibility of integrating cell external factors (diseases, age, hepatic and renal functions, environmental characteristics) that are imperative for the whole organism function. Although, extrapolation of *in vitro* results to humans must be done very carefully²⁰⁸, the *in vitro-in vivo* correlation represents an important tool for prediction of *in vivo* pharmacokinetics. Clark *et al.* in 2011 tried to compare results obtained *in vitro* with those *in vivo* for a preclinical evaluation of a microbicide in the form of a micronized and non-micronized drug. However, *in vivo* pharmacokinetic results (in rabbits) failed to corroborate the higher permeability for the micronized drug obtained with reconstructed tissue *in vitro* studies²⁰⁸.

Drug physicochemical and biopharmaceutical properties as well as physiological and environmental organism characteristics should also be considered. Among them are drug solubility, acid dissociation constant (pKa), drug permeation, octanol-water partition coefficient (logP) and the environment fluid pH²¹³. Therefore, *in vitro* permeability studies also play a key role in determining a formulation strategy in order to assist solubility, dissolution and stability²¹⁴.

In the 90's Gorodeski *et al.* developed a method based on the harvesting of cervical-vaginal cells collected from women, aged from 22 to 49 years, undergoing hysterectomy. Initially, a primary cell culture of human ectocervical epithelial cells was obtained (hECE)²¹⁵, that included histologically normal cells only. This technique was applied to permeation mechanistic studies. To this purpose, the cells were grown on collagen-coated ceramic-based filters and differentiated into a 5-12 cell layers to form a squamous stratified epithelium, mimicking the biological characteristic human cervical-vaginal epithelium^{216,217}. When these cells were compared to a cervical cell line (CaSki), the latter ones showed to be simpler to maintain and a better alternative to the primary cultures obtained from hysterectomy specimens. In this study, it was also evidenced the epithelial nature of CaSki cells and their improved differentiation when grown on filter support²¹⁸. Although they are easier to grow, maintain and test, cell line cultures correspond to immortalised cells and consequently may not mimic real cell behaviour. From this point of view, primary cell cultures should be valued since they represent a more reliable biologic model.

When different cellular models were developed and compared, hECE, the primary culture, was settled as a model for ectocervical epithelium, while ECE16-1 and CaSki, both immortalised cell lines, expressed phenotypic characteristics of squamous metaplastic cervical epithelium and endocervical epithelium respectively²¹⁹. Later on, CaSki endocervical cell line was shown to be able to form cell monolayers and even bi/trilayers, depending on cell seeding density, and was proven to be useful for transport mechanisms studies²²⁰⁻²²². These experiments resulted in interesting findings about the importance of tight junctions and their influence on transepithelial electrical resistance (TEER)²¹⁷ and the increase of TEER by seminal fluid²²¹. They also contributed to improve the knowledge about the modulation of vaginal permeability to pyranine (used as a model drug for paracellular transport) by factors such as estrogen stimulation and aging (due to changes in the resistance of both the lateral intercellular space and the intercellular tight junctions)²²³⁻²²⁵, or extracellular ATP and Ca^{2+} (due to their effects at the tight junctions level)^{218,226}.

Permeability studies using either primary cultures or cell lines of vaginal/cervical epithelial cells have been described in the literature and are herein discussed^{223,225,227-229}. Cell lines represent more standardized models than primary cultures, since they are ready to use without rising important inter laboratory variability results and interpretations. However, cells permeability is higher than explants. As it has not been evidenced the difference in permeability between primary cell cultures and cell lines but, this topic deserves to be studied and explored, for example using the TEER measurement. The simplicity of these *in vitro* models can lead to limitations in terms of *in vitro-in vivo* data correlation when compared to methods based on human vaginal and cervical tissue explants. The importance of establishing these correlations justifies the need to proceed with tests that better mimetize the corresponding *in vivo* mechanisms, which may be made by using reconstructed tissue, tissue models (*ex vivo*) and animals (*in vivo*).

Regarding tests using reconstructed tissue, the Epivaginal® model (MatTek Corporation, Ashland, United States of America), is a commercial vaginal-ectocervical (VEC) tissue-like model widely used for microbicides testing. It consists of a 3D-culture of non-transformed human vaginal-ectocervical epithelial cells grown on polycarbonate cell culture tissue inserts. The VEC tissue model has proven to be highly reproducible and represents a non-animal method to assess the irritation effect of contraceptives, microbicides, and vaginal-care products^{190,211}. Despite being largely used for studying prevention of pathogen transmission^{230,231} and tissue toxicity¹⁹⁰, the complexity of the multi-layer structure makes it a promising option for permeability studies. Several other authors have already used EpiVaginal® as model to predict vaginal permeability to chemicals^{232,233}.

1.5.3.2.2. *Ex vivo* models

Ex vivo models have been used for predicting drug permeation through the vaginal barrier by using either human or animal vaginal tissue. *Ex vivo* tissues allow not only for drug permeation assays but also enables histological analysis to assess epithelial differences before

and after drug application/permeation²³⁴. While some authors may classify these *ex vivo* experiments as *in vitro* experiments, the two concepts are considered to be different for the purpose of this thesis. Laboratorial reconstructed tissues are herein classified as *in vitro* assays, as they are grown in artificial milieu, while *ex vivo* assays require tissue excision from animals or humans, and their use in conditions that preserve their original biological and physical characteristics for a certain period.

Fresh human cervical explants are clinically obtained from women undergoing planned hysterectomies. In the USA some repositories provide fresh samples to research institutes and industries, nevertheless it is doubtful that the samples available are sufficient to support extensive experiments, such as the toxicological ones⁴². Researchers are concerned with the difficulty to obtain and maintain human explant specimens at the laboratory. To overcome these difficulties, freezing of specimens has been considered and no negative influences in permeability parameters were found for this tissue conservation method. In fact, no statistical differences were found between fresh and frozen tissue permeability results when the later was collected 1 hour after the surgical excision, transported in PBS or culture media at 5°C, frozen with methanol/dry ice or liquid nitrogen, and kept frozen at -80°C¹⁷. Previous studies conducted by van der Bijl *et al.* showed that freezing at -85°C had no effect on tritiated water permeation through vaginal tissue, when this has been kept in a transport fluid, and transferred to laboratory within 1 hour, snap-frozen in liquid nitrogen and stored at -85°C for up to 6 months²³⁵⁻²³⁹. Although, permeability studies may not be influenced by tissue freezing-thawing, the impact on vaginal irritation is not clear, since cellular viability might be affected^{42,240}. Meanwhile, the difficulties encountered with the human tissue samples impelled the demand for other alternatives, such as animal tissues.

Vaginal tissue obtained from different animal species such as rodents, rabbits, monkeys, cows and sheep have been used for permeability studies²⁴¹⁻²⁴⁵. Vaginal absorption may be significantly different when comparing animal and human models. Bovine vaginal tissue was used to test drugs permeation, as surrogate for human tissue due to anatomical and physiological similarities²⁴⁵. Nonetheless, bovine epithelium exhibits significant histological differences when compared with human vaginal epithelium and is not widely applied. In contrast, porcine tissue represents the animal model most commonly applied for this purpose. Porcine *ex vivo* tissue specimens are convenient, since they are simple to handle, inexpensive to obtain and easy to work comparing to the whole animal. Human and porcine vaginal tissues present substantial histological similarities^{34,240} (stratified squamous epithelium supported by connective tissue - **Figure I. 2**). Additionally, porcine vagina can be easily accessed through local slaughterhouses.

Although, in general, porcine vaginal tissue seems a good *ex vivo* permeability model for human vaginal tissue extrapolation it is of great importance to validate a permeability study concerning other tissues or *in vitro* tests. It has already been shown that for hydrophilic molecules, (water and vasopressin, for example), the porcine vaginal tissue is an accurate *in*

vitro permeability model of human tissue, however for more lipophilic molecules (such as oxytocin) the flux through porcine vaginal tissue was 53% higher than the corresponding estimated value through the human vaginal tissue²⁴⁶. *Ex vivo* tissues are mainly involved in drug permeation testing by using one of three established techniques: Franz cell systems, flow through diffusion cell and Ussing chambers.

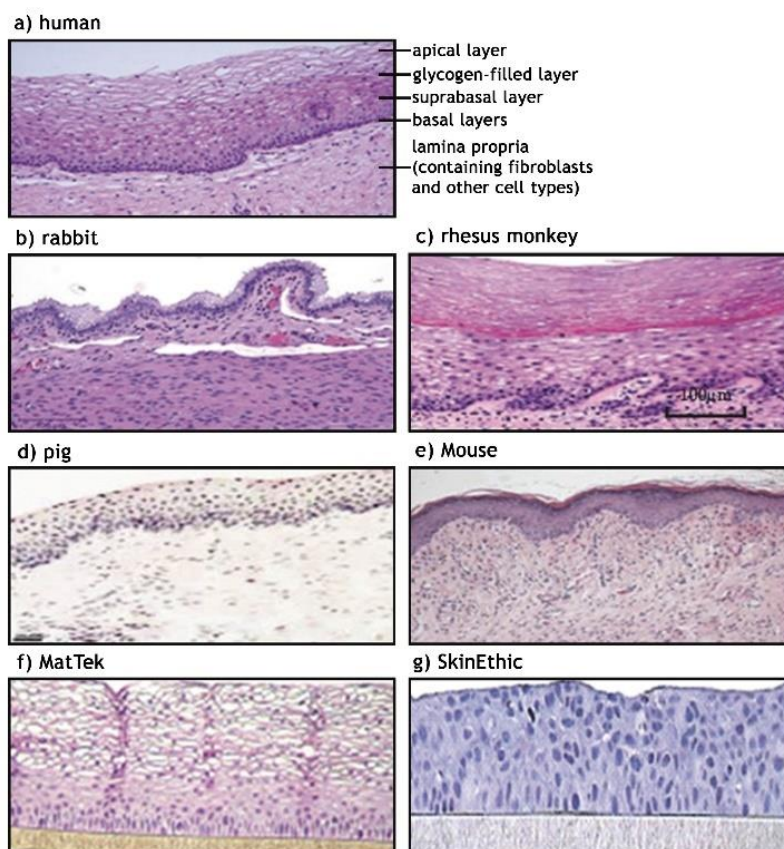


Figure I. 2: Comparison between H&E-stained vaginal epithelium of: human (a), rabbit (b), rhesus monkey (c), pig (d), mouse (e), EpiVaginal from MatTek Corporation (Ashland, MA, USA) (f) and HVE – Human vaginal Epithelium from SkinEthic (Nice, France) (g). Reproduced from⁴² with kind permission from FRAME.

I.5.3.2.3. *In vivo* models

In vivo models represent the most complete approach to achieve experimental data. However, they are associated with ethical issues, require high cost expenditures and are time consuming. Vaginal permeability testing is scarce on *in vivo* studies, although it could be of great interest especially for systemic drug delivery systems, provided that it is previously validated, ethic, safe and have clear benefit to new drug and formulation development. Acartürk *et al.* used normal and ovariectomized rabbits to mimetize different reproductive physiological status, particularly post-menopausal human vagina. Explants of vaginal tissue from these animals were further used for comparative *in vivo*/ *ex vivo* vaginal permeability and enzymatic activity studies²⁴⁷. This model was also applied in the study of nonoxynol-9 as spermicide²⁴⁸. More recently, the pig model has also been used for *in vivo* experiments. Female large white pigs were used for the determination of zidovudine and polystyrene sulfonate in

plasma and vaginal tissue, after the application of an intravaginal bioadhesive polymeric device for up to 28 days. At the end of this period, vaginal tissue was collected for histological analysis, and for substances extraction. Both zidovudine and polystyrene sulfonate were found in low concentrations in plasma, indicating the high retention in the vagina. Additionally no histopathological toxicity was evidenced for this intravaginal bioadhesive polymeric device²⁴⁹.

I.5.3.2.4. Release and permeation systems

The United States Pharmacopeia (USP) clearly defines the performance tests for topical drug products, including vaginal administration products, which are focused on the assessment of *in vitro* drug release. It is defined that this test must be performed in vertical diffusion cell (VDC) systems, which by their simplicity can provide reliable and reproducible measurements of drug release from semisolid formulations. Franz cells represent the most often used VDC, on the top of which 200-400 mg of the testing formulation is placed, over the membrane to be studied. The application site can be variable in size, but it has typically 15 mm in diameter. For vaginal drug delivery systems the assay temperature must be kept at $37\pm1^{\circ}\text{C}$. Samples of the recipient fluid are collected up to 4 - 5 hours, and the volume withdrawn is replaced with fresh medium. Sink conditions must be assured, meaning that the receptor medium must have a high capacity to dissolve the drug, and the receptor media should not exceed 10% of the concentration of the standard at the end of the test²⁵⁰. Moreover, FDA's Guidance for Industry on Scale Up and Post Approval Changes for Semisolid (SUPAC-SS) dosage forms describes the release rate studies using the vertical diffusion cell (Franz cell) procedure and requires *in vitro* release rate comparison between prechange and postchange products for approval of SUPAC-related changes²⁵¹.

Although USP recommendations point towards the use of synthetic, inert and highly permeable membranes in the context of these drug release studies, the interest in performing *in vitro* permeability studies using biological membranes is evident. Biological tissues/membranes are more similar to the *in vivo* conditions and may provide information on bioavailability of the drug. However, they are also more complex, and the obtained data can be more difficult to interpret and discuss. Generally, the difficulties to be encountered are related to tissue preparation and system setting-up. The membrane preparation depends on tissue provenience. Since human tissues are difficult to obtain, frozen and thawed samples are frequently used following previous studies that demonstrated that this procedure does not affect tissue permeability^{238,252}. Permeation systems intended to study vaginal drug release from vaginal dosage forms include Franz cell systems, flow through diffusion cells and Ussing chambers.

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental institution which harmonises policies in developed countries and has issued several publications concerning the safety assessment of chemicals and chemical preparations. Since, for example, vaginal lubricants can be classified as medical devices or even hygiene (cosmetic) products, for marketing purposes, (i.e. they are not medicines, and so do not

necessarily follow the pharmacopeia specifications), manufacturers should follow the same methodology on characterizing products to standardize procedures. Standardization of the manufacture and testing methods provide better awareness of the product, even on already commercialized products¹⁵⁹. The OECD series on Testing and Assessment Number 28, clearly describes for skin permeability testing, the type of studies (*in vivo* or *in vitro*) to be used and their detailed procedures. These include the species to be selected together with the number, sex, housing and feeding conditions of animals used in *in vivo* studies; and for *in vitro* studies the diffusion cells type, the characteristics of receptors fluids and skin preparation. Additional considerations regarding the testing substance are also described. However, no specific guidelines are available for vaginal (or genital) products. Since the vaginal route plays an important role both on local and systemic delivery, the availability of validated vaginal models and regulatory recommendations similar to those for skin permeability studies is of extreme relevance.

I.5.3.2.4.1. Franz cells

Several research *in vitro* studies have already been conducted to characterize the vaginal barrier in terms of drug permeation using a Franz cell system^{253,254}. Additionally, this model constitutes the methodology of reference in the USP for topical drug release. The system is composed of two compartments: a donor compartment (upper chamber) and a receiver compartment (lower chamber) (**Figure I. 3**). A membrane is placed between the two compartments. The formulation to be tested is placed on the top of the membrane, in the donor compartment which does not need to be filled with a liquid. Heated water circulation is maintained around the receptor chamber all over the process, usually at 37°C to mimic human body temperature. The stirbar guarantees the receptor solution homogeneity. Samples can be accessed over time by collecting aliquots through the sampling port.

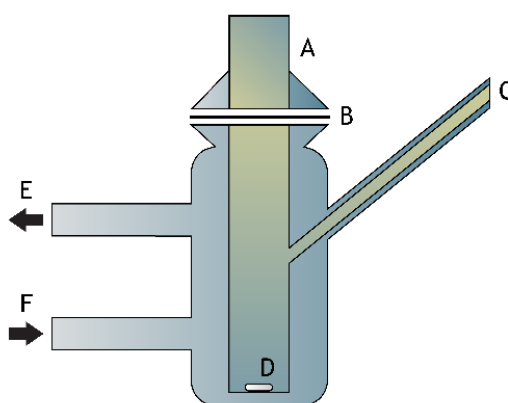


Figure I. 3: Schematic representation of a Franz cell. (A) donor compartment; (B) membrane; (C) sampling port; (D) stirbar; (E) water outlet; (F) water inlet.

A phosphate-buffered saline (PBS) solution with physiological pH is the mainly used media in the receptor chamber^{17,255} despite oxygenated Ringer buffer has also been used²⁴⁵. Tissue thickness and area must be taken into consideration in results calculations. USP recommends

the use of synthetic and inert membranes in which the product to test is placed, letting the system to saturate for 30 minutes (equilibration period). Aliquots from the receptor compartment are collected at predefined time intervals. The aliquots volume withdrawn must be replaced with the same volume of receptor solution to ensure *sink* conditions. Afterwards, aliquots should be analysed through diverse analytical techniques for identification of drug in the collected sample. Membrane/tissue should also be checked for histological modifications after the permeability study. Steady-state flux (J_{ss}) and apparent permeability coefficient (P_{app}) can be calculated based on the Fick's First Law of Diffusion²³⁵.

The permeability chamber design may be responsible for results' variability. Classic Franz cell system used in permeation studies can lead to stasis and/or accumulation of perfusate in the receptor chamber, which can be overcome by using a diffusion flow-through system²⁵⁴. Concerning the experimental conditions, temperature stands as an essential parameter to be controlled since drug diffusion depends on temperature. Also, the origin of tissue and the experiment duration must be optimized to get the best profit of permeability studies³⁴.

1.5.3.3. *In vitro* drug release

Dissolution testing in the pharmaceutical industry has been employed as a fundamental tool both in formulation design and quality control^{251,256-261}. It was initially developed for immediate release but then it was extended to controlled/modified release and other dosage forms. Recently, dissolution testing was widened to a variety of "novel" or "special" dosage forms such as suspensions, orally disintegrating tablets, chewable tablets, chewing gums, transdermal patches, semisolid topical preparations, suppositories, implants and injectable microparticulate formulations, and liposomes²⁶¹. In terms of terminology, for non-oral dosage forms (topical and transdermal delivery systems, and suppositories), the test is referred as "drug release" or as "*in vitro* release". It is worth noting that in general, an *in vitro* dissolution/release test is expected for each novel/special dosage form, regardless of whether the intended effect is systemic or non-systemic (eg, topical semisolid dosage forms)²⁶¹.

The selected membrane should provide an inert holding surface for the test formulation, but not a barrier, so that the drug release would reflect the vehicle properties and not the membrane rate-limiting properties.

In vitro release is one of several standard methods which can be used to characterize performance characteristics of a finished topical dosage form, i.e., semisolids such as creams, gels, and ointments. Important changes in the characteristics of a drug product formula or of the thermodynamic properties of the drug(s) it contains should show up as a difference in drug release²⁶². Release is theoretically proportional to the square root of time (\sqrt{t}) when the formulation in question is in control of the liberation process because the release is from a receding boundary²⁵¹.

The drug in the dissolved or suspended state in the vehicle has to diffuse and be released by the vehicle, becoming theoretically available to penetrate into different epithelia. For quality control purposes, the method can be employed as performance test designed to

demonstrate that the dosage forms were manufactured according to specifications and all critical manufacturing steps assuring batch-to-batch sameness. Secondly, in research and development the focus of release testing is shifted to other purpose. The vehicle composition and design strongly influence the product performance and the speed of the drug release and ability to permeate biological membranes²⁶³.

I.5.3.4. Quantification methods development and validation

To accomplish drug quantification for the abovementioned methods, a bioanalytical tool should be conveniently developed, optimised and validated. Bioanalysis includes the set of analytical techniques and procedures applied in the characterisation and quantitative determination of drugs in various matrices²⁶⁴.

Firstly, the analytical conditions must be optimized, in order to a proper validation could consequently be performed. Only this step of validation can provide accurate, precise and reproducible data throughout study-sample analysis. The method validation is the systematic process of defining the analytical requirements, confirming that the method under consideration has performance capabilities consistent with the demands of its intended application²⁶⁵.

High Pressure Liquid Chromatography (HPLC) represents nowadays the standard quantitative methodology, being able to separate and quantify mixtures of compounds with low and high molecular weight, as well as different polarities and acid-base properties in a variety of matrices²⁶⁶. The development of a bioanalytical method is unique and specific for each drug candidate and it depends, among various factors, on the analyte physicochemical features (e.g. molecular weight, pKa and lipophilicity), nature of the matrix, sensitivity required and the available instrumentation. Chromatographic analysis intends the separation of compounds in a sample with good resolution, without interferences and within a reasonable running time. In general, the HPLC separation of the analytes is accomplished based on the differences in affinity of the compounds between two phases: one mobile and the other stationary. Since the majority of the drugs and metabolites are polar in nature, the reversed-phase chromatography is the most commonly applied method in which the mobile phase is polar, composed of a mixture of water/buffer and organic solvents (acetonitrile or methanol); and the stationary phase is non-polar, consisting of hydrocarbon chains chemically attached to the silica base packing of the column (e.g. C18 and C8). Hence, while polar compounds get eluted firstly, the non-polar ones are retained in the column for a longer period of time and therefore, the separation of the analytes is sorted according to their increasing molecular hydrophobicity. Furthermore, depending on the number and polarity of the compounds to be resolved or separated, the mode of elution can be stipulated as isocratic or gradient²⁶⁷. Additionally, buffer solution could be needed when an analyte is ionic or ionisable under reversed-phase conditions (pH values within the range 2-8)²⁶⁶. The flow rate and temperature of analysis may likewise have quite significant effects on the analytes' resolution and retention time. The choice of the most convenient system for the detection of the eluting components is dependent on the

analytes' properties. Different types of detectors, such as ultraviolet (UV), fluorescence and mass spectrometry, are available to be used in HPLC analysis. Even though, in general terms, either fluorescence or mass spectrometry provide better sensitivity and selectivity than UV detection, but the latter is cheaper, more easily accessible and presents a broad spectrum of application²⁶⁸.

Owing to the undeniable importance of method validation in whole field of bioanalysis, it was felt the need to standardise the overall validation parameters and acceptance criteria by globally establishing appropriate guiding principles. Since the 90s, several workgroups have addressed this issue, and published relevant reports²⁶⁴ which subsequently, served to establish the general recommendations on bioanalytical method validation²⁶⁹. Also, the European Medicines Agency (EMA), published in 2011 a similar document to facilitate regulatory issues on the European countries²⁷⁰. Furthermore, the International Conference for Harmonization has also contributed to this area of validation by publishing the topic Q2 (R1) "Validation of Analytical Procedures: text and methodology".

All three agencies recommend that the method validation should conduct a deep investigation concerning parameters such as selectivity, linearity, precision, accuracy, limit of quantification and limit of detection, recovery and stability; and even state the limits of acceptance for each parameters.

Concerning the method validation, it comprises three different levels whose application depends on the circumstances in which the method was developed and how it is going to be used: full validation, partial validation and cross validation²⁶⁹. A full validation is required when the method is implemented for the first time or a new drug/metabolite is included. However, in situations where minor alterations are made to a previously validated method, such as transfer between laboratories or analysts, changes in equipment, in species within matrix, in matrix within species, in sample processing procedures, in storage conditions, etc., a full validation may not be necessary and therefore, a partial validation can be performed ranging from as little as one intra-assay accuracy or precision determination to an almost full validation²⁶⁹. A cross validation consists of a comparison of the validation parameters when two or more bioanalytical methods are used to generate data within the same or across different studies conducted in distinct laboratories.

I.6. Aims of this thesis

Although there are several formulations commercially available for vaginal use, both for therapeutic purposes and for cosmetic/hygienic or medical devices actions, preclinical data on its potential to cause acute irritation and toxicity is not widely addressed. The global aim of this thesis is to establish methods, from simple technological characterization to complex ones, such as *ex vivo* permeation, that could be predictive at early stages of formulations development of their *in vivo* performance. To achieve this, a collection of ten semisolid vaginal medicines, globally available, was included in this study. Furthermore, two formulations, that do not contain active substances, but are widely described on the literature, were used as controls.

Specifically, it is intended to:

- Develop, improve and apply a complete technological characterization methodology for vaginal semisolids, from a safety and performance perspective, considering their pharmacotechnical profile;
- Optimize cell-based and organ-based (*in vitro* and *ex vivo*) models to test the formulations toxicity, while applying techniques capable of predicting their acute irritant potential;
- Perform drug delivery studies, including *in vitro* release and *ex vivo* permeation of the active substances included in the formulations to refine experimental setups and generate data for comparison with other products.

Moreover, during the deployment of this work, the potential for knowledge transfer for the pharmaceutical industry was also a core objective. Since this thesis is part of a university-company partnership, the methods herein explored are expected to fit the processes of new products development *in-house* or as services provided to the pharmaceutical, medical devices and cosmetic industries.

CHAPTER II

TECHNOLOGICAL CHARACTERIZATION: CHEMICAL, PHYSICAL AND PHYSIOLOGICAL METHODOLOGICAL INSIGHTS

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II.1. GENERAL CONSIDERATIONS

Several formulations for vaginal administration are available in the market as medicines or OTC (Over-the-Counter) products, some of them classified as medical devices or cosmetics. However, especially for the later ones, there are few data on their potential to cause acute irritation and toxicity, or their suitability is poorly supported, particularly because preclinical assays cannot be performed on animals and clinical trials are not usually considered.

Among the semisolid vaginal dosage forms creams and gels have gathered particular interest among researchers engaged in developing vaginal formulations¹⁶. They have been pointed as the most preferred dosage forms among women although leakage has been frequently referred as their major drawback. These formulations may require multiple daily administrations in order to obtain the desired therapeutic concentration and to provide a uniform distribution of the drug^{5,49-51}.

To overcome this limitation, major research in this field has been focused on bioadhesive polymers or novel dosage forms, but by now only limited evolution has been found in commercial vaginal products. Those strategies are, however, expected to increase residence time, improving both efficacy and safety of the new delivery systems.

Technological properties of vaginal semisolids have been considered to correlate with their *in vivo* performance regarding safety and efficacy. This is the case for the ability of formulations to maintain the normal vaginal pH²⁷¹, to be isosmolal¹⁵⁹ and to improve the retention through adhesion measurements¹⁷³. Not only should researchers aim for better products but also to develop selective tools (i.e. characterization methods) to characterize the products more precisely¹⁵¹, and so, better predict their performance. Also, new methods should address physiologic featurings in perspective of earlier cognizance of *in vivo* possible effects. Ultimate acceptability and clinical efficacy of such preparations require that they possess optimal mechanical properties (ease of removal from the container, spreadability over the substrate), rheological properties (viscosity, elasticity, thixotropy, flowability), and other desired properties such as bioadhesion, desired drug release, and absorption¹⁵². Nevertheless, still very little attention has been given to the influence of the vaginal environment on formulation performance. Actually, formulations testing is generally carried without considering the effect of physiologic parameters such as temperature and solvency in vaginal fluids. It is well known that these variables can impact on formulations properties, especially, pH, osmolality, rheology and ultimately bioadhesion. For example, Aka-Any-Grah *et al.* have performed characterization studies using dilutions in simulated vaginal fluid to determine final rheological profiles and bioadhesion in early steps of formulations development and concluded that this tool is supposed to predict more accurately the *in vivo* behaviour¹⁰⁹.

This chapter pretends to characterize several products already marketed for vaginal administration, using well established methods and also proposing new methods and approaches to gather primary information of the formulations behaviour when in contact to physiologic vaginal fluid.

II.1.1. Objectives

The objective of this part of the work is to develop and apply a characterization methodology for vaginal semisolid formulations in view of safety and performance considering physiological parameters. Specifically, it is intended to:

- Determine the organoleptic characteristics of the formulations in study;
- Perform pH and acid-buffering capacity assays on vaginal fluid simulant;
- Disclose formulations osmolality, and even its osmolality after a physiologic dilution;
- Evaluate formulations textural characteristics through firmness and adhesiveness;
- Characterize formulations viscosity directly and diluted in physiologic fluids;
- Perform bioadhesion evaluations on a mechanical *ex vivo* model.

II.2. EXPERIMENTAL

II.2.1. Materials and Methods

II.2.1.1. Tested products

The semisolid products tested in this study are classified as medicines and were acquired in Portuguese community pharmacies, being also commercialized across the EU and USA (Table II. 1). Some of these are Over-the-counter (OTC) products, not requiring a medical prescription to be dispensed at pharmacies (Gino-Canesten® and Gyno-Pevaryl®). The majority of these dosage forms are creams, except Blissel® that is a gel. All products are intended for vaginal use except Colpotrophine®, which is recommended by the manufacturer for vulvar application. Universal placebo gel^{99,158,233,272} and Replens®^{14,93,166,173} were included, since they represent widely studied formulations. The comprehensive and attentive review on vaginal products classification showed us that there is misunderstanding between vaginal and intravaginal products. We assume that when a product is intended for vaginal application it means intravaginal administration, and not for external/vulvar application, like the case of Colpotrophine®. This term inaccuracy is specially noted in the case of vaginal solutions, which should fit up for intravaginal irrigations, and that in the majority of the cases are indicated for external washes.

II.2.1.2. Materials

Vaginal fluid simulant (VFS) was prepared as described by Owen and Katz in 1999: sodium chloride 3.51 g (*JT Baker*, United States of America), potassium hydroxide 1.4 g (*VWR Prolabo*, France), calcium hydroxide 0.22 g (*Acros Organics*, United States of America), Bovine Serum Albumin 0.018 g (*Sigma*, Germany), lactic acid 2.00 g (*Sigma*, Germany), acetic acid 1.00 g (*Fischer Scientific*, United States of America), glycerol 0.16 g (*Acofarma*, Spain), urea 0.4 g (*VWR Prolabo*, France) and glucose 5.00 g (*VWR Prolabo*, France) were added to 1 L of milliQ water and stirred mechanically until complete dissolution³⁷. The pH of the mixture was then adjusted to 4.5 using hydrochloric acid, and the final volume was adjusted to 1 L. All other chemicals and reagents were of analytic grade or equivalent. For the modified VFS (VFS_m) a 1.5% (w/w) concentration of porcine gastric mucin type II (*Sigma*, Germany) was used²⁷³.

II.2.1.3. Organoleptic characteristics

The organoleptic characteristics studied were: general aspect, homogeneity, colour, odour, feel to touch.

Table II. 1: Studied formulations general characteristics (information provided by the manufacturer).

Therapeutic action	Trade name/labelled dosage form	API	Excipients	Marketing Authorization Holder (in Portugal)
Antifungals	Gino-Canesten®, vaginal cream	Clotrimazole 10 mg/g	Benzyl alcohol, Cetyl palmitate, Cetostearyl alcohol, Purified water, Polysorbate 60, Sorbitan monostearate, Octyldodecanol Ethyleneglycol and polyethyleneglycol, Stearate palmitate, Saturated glycerides polyglycolized, Glycerol isostearate, Liquid paraffin, Methylparaben, Sorbic acid	Bayer Portugal, S.A.
	Sertopic®, vaginal cream	Sertaconazole 2 g/ 100 g	Tefose 63, Labrafil, Peceol, Liquid paraffin, Nipagin, Sorbic acid	Ferrer Portugal, S.A.
	Dermofix®, vaginal cream	Sertaconazole 20 mg/g	Stearate pegoxol 7, Liquid paraffin, Oleic macrogolglycerides, Butylhydroxyanisole (E320), Benzoic acid, Purified water	Azevedos Laboratories - Pharmaceutical Insustry, S.A.
	Gyno-Pevaryl®, vaginal cream	Econazole 10 mg/g	Propylene glycol, Hydrogenated lanolina, Sweet almond oil, Polyglycol esters of fatty acids, Cetyl alcohol, Glyceryl monostearate, Sodium EDTA, Purified water	Johnson & Johnson, Lda.
	Lomexin®, vaginal cream	Fenticonazole 20 mg/g	Polysorbate 60, Sorbitan stearate, Cetostearyl alcohol, Thick paraffin, White Vaseline, Purified water	Recordati Ireland Ltd.
	Gino Travogen®, vaginal cream	Isoconazole 10 mg/g	Purified water	Bayer Portugal, S.A.
Antibacterials	Dalacin V®, vaginal cream	Clindamycin 20 mg/g	Propylene glycol, Cetostearyl alcohol, Liquid paraffin, Sorbitan stearate, Benzyl alcohol, Cetyl palmitate, Stearic acid, Polysorbate 60, Purified water	Pfizer Laboratories, Lda.
Oestrogens	Ovestin®, vaginal cream	Estriol 1 mg/g	Octyldodecanol, Glycerol, Cetyl alcohol	Aspen Pharma Trading Ltd.
	Blissel®, vaginal gel	Estriol 50 µg/g	Stearyl alcohol, Polysorbate 60, Sorbitan stearate, Lactic acid, Chlorhexidine hydrochloride, Sodium hydroxide, Purified water, Synthetic spermaceti Glycerol (E 422), p-hydroxybenzoic acid methyl ester, p-hydroxybenzoic acidpropyl ester, Polycarbophil, Carbomer, Sodium hydroxide, Hydrochloric acid, Purified water	ITF Medivida, Pharmaceutical Products, Unip. Lda.
	Colpotrophine®, vaginal cream	Promestriene 1 g/ 100 g	p-hydroxybenzoic acid methyl ester, p-hydroxybenzoic acid propyl ester, Mono- and diglycerides of saturated fatty acids, Polyglycol ether of saturated fatty alcohols, Oleic acid decyl ester, Triglycerides of capric and caprylic acids, Glycerol, Purified water	Teva Pharma - Pharmaceutical Products, Lda.
Controls (Reference products)	Universal placebo, vaginal gel	Not applicable	Purified water, Hydroxyethylcellulose, Sodium chloride, Sobic acid, Caramel color, Sodium hydroxide	Not applicable
	Replens®	Not applicable	Purified water, Polycarbophil, Paraffin oil, Glycerin, Palm oil hydrogenated, Carbomer homopolymer type B, Sorbic acid, Sodium hydroxide	Lil'Drug Store Products, Inc. (in France)

II.2.1.4. pH and buffering capacity

For the pH determination, the probe (for viscous products, InLab Viscous, *Mettler Toledo*, USA) was directly immersed on the formulation (Seven Compact, *Mettler Toledo*, USA). The pH-buffering capacity was accomplished by titration with NaOH 1 N added in increments of 20 μ L to the dispersion of formulations in Normal Saline - NS (NaCl 0.9%) or VFS until $\text{pH} \geq 9$ ¹⁵⁹. The relevant and absolute buffering capacity were determined using the best fit linear model - *Curve Expert Version 1.4* (Copyright 2013, Daniel G. Hyams). The absolute buffering capacity was calculated and defined as the amount of sodium hydroxide needed to change by one unit the initial pH value. The relevant pH-buffering capacity was calculated as the amount of sodium hydroxide required to reach a pH value of 5 (higher than the normal vaginal pH of reproductive aged women)^{157,274}. For products which had initial pH higher than 5, the reverse buffering capacity was performed by adding 20 μ L of HCl 1 N until pH was lower than 3.

II.2.1.5. Osmolality

The osmolality was determined in triplicate using a freezing point osmometer (Osmomat 3000, *Gonotec*, Germany), as previously described^{159,166}, on a 50 μ L aliquot. The standardization was performed using three standards: distilled water (zero point), NaCl 300 mOsm/Kg and NaCl 850 mOsm/Kg, commercially available from the equipment manufacturer. Furthermore, osmolality was determined in a mixture with vaginal fluid simulants. An amount of each product corresponding to the daily dose (measured with the proprietary applicator) was diluted in 0.75 mL of VFS or VFS_m. This procedure was established to estimate the osmolality of the product when put in contact with vaginal fluid simulants, since 0.75 mL is the estimated mean volume of fluid present in the vagina at any moment^{37,49,109}.

II.2.1.6. Texture: Firmness and Adhesiveness

Texture analysis included adhesiveness (N.mm) and firmness (N) determinations using a texturometer (TAXT Plus, *Stable Micro Systems*, United Kingdom). These two parameters were determined in the same run, using a cylindrical probe with a diameter of 10 mm (P10)^{169,170} in compression mode and «return to start test». The maximum positive force (N) to penetrate the formulation for 5 mm was registered and corresponds to the formulation firmness (pre-test speed, test speed and post-speed: 3 mm/s). Force measurements required to detach the probe from the formulation during the returning movement allowed for the calculation of the work of adhesion, which is herein described as adhesiveness. Measurements were performed, in triplicate, at room temperature, complying with the laboratorial and equipment manufacturer protocol.

II.2.1.7. Bioadhesion

Formulation adhesiveness to the biological substrate (porcine vaginal tissue) was assessed using the texturometer (TAXT Plus, *Stable Micro Systems*, United Kingdom). The method employed consisted on a mechanical approach to bioadhesion since it is based on the evaluation of tensile strength of the interfacial layer formed between the formulation and the vaginal epithelium¹⁷³. The vaginal epithelia was excised from porcine vaginal tubes (obtained from approximately 6 months' year old animals, kindly conceded from a local slaughterhouse). The vaginal tubes were cut longitudinally, washed with Hank's Balanced Salt Solution (HBSS) pH 4.2, wrapped in aluminium foil, and preserved in an air tight bag at -20°C. For the experiment, vaginas were thawed at room temperature in HBSS. The epithelium samples were fixed using a mucoadhesion rig which was placed on the equipment's base. The whole system composed of the mucoadhesion rig with the tissue and the probe with the formulation was kept at 37±0.5 °C in an oven. The tissue was hydrated with 50 µL of VFS_m, since mucin is the protein most likely to be responsible for bioadhesion. A double-sided adhesive tape was used to attach a small piece of cellulose acetate membrane to the probe, where 30 mg of formulation were adsorbed (the formulation was weighted directly on the probe). Cellulose acetate membrane without formulation was used as control. The software was used in adhesive mode. The pre-test speed was 0.5 mm/s with a trigger force of 0.02942 N to allow for sensitive detection of the tissue. Post-test speed was 0.1 mm/s¹⁰⁰. The contact/hold time was 3 min, and the force applied was 2.5 N²⁷⁵. The force of detachment was recorded as well as the graphical negative area, representative of the work of adhesion (N.mm) necessary to unbind the two surfaces. One-way ANOVA statistical test with multiple comparisons was applied to denote differences ($p < 0.05$) between the control and formulations (GraphPad Prism 6.0).

II.2.1.8. Viscosity

Viscosity was assessed using a cone-plate rheometer (Brookfield DV-3T, *Brookfield*, USA). Viscosity measurements were performed at room temperature (25°C) and at vaginal physiologic temperature (37°C), using plain formulations and after diluting in VFS. Cone spindles used were CPA-52Z and CPA-40Z (*Brookfield*, USA), both required a 0.5 g or 0.5 mL sample, and had 3° and 8°, and 1.2 cm and 2.4cm, cone angle and radius, respectively. To assess the thixotropic behaviour of formulations, a range of test speeds between 5 and 200 RPM was established (torque 10-100%). Tests were performed in triplicate during 1 min, and the formulation was left to rest for 1 min between measurements²⁷⁶. Formulations' dilution to physiologic assemblies were performed as described on the osmolality section.

Furthermore, to study rheological modifications following application, an *ex vivo* model for administration was developed using porcine vaginas. For this purpose, a daily dose of all the formulations was placed, with the help of the proprietary applicator, onto an excised vagina obtained from a local slaughterhouse. The organ was isolated from the upper part of the remaining reproductive system, placed on a tray, and 0.75 mL of VFS were inserted into the

vagina. Then, the trays were placed in an incubator at 37°C with mild agitation (50 RPM). After 3 h the trays were removed and the vaginas were opened longitudinally for observation and collection of the formulation which was further assessed concerning viscosity.

II.2.1.9. Data processing and statistical analysis

Data was analysed to produce arithmetic means with standard deviations (SD) using Microsoft Excel. Analysis of variance (ANOVA) was performed to determine the significance of the difference between sets of data ($p < 0.05$) using GraphPad Prism 6.0 (Graph Pad Software, USA).

II.2.2. Results and Discussion

II.2.2.1. Organoleptic characteristics

Organoleptic characteristics were determined taking into account both users' perspective and formulation suitability for vaginal administration. Thus, evaluated parameters comprised colour, odour, texture and homogeneity. As previously described women prefer vaginal products presented as semisolids, odourless and colourless, either being gels, creams or ointments^{277,278}. Also, women would rather privilege natural origins for drugs/excipients, and application by means of an applicator²². In terms of pharmaceutical dosage form the majority of the studied products are labelled as vaginal creams, except for Blissel® and Replens® that are presented as gels. These gels are both colourless. None of the formulations showed strong odours. Also, all of them had a soft texture and were homogeneous, being in accordance with female users expected preferences²⁷⁷. All products, except Colpotrophine®, are marketed with disposable applicators, in order to allow for a more comfortable application. These characteristics are also in accordance with women's expectations reported in previous studies for determination of the ideal organoleptic characteristics for microbicides using personal interviews¹²⁸ and focus groups²⁷⁹. Regarding the clinicians' perspectives, it has been published that both general practitioners and gynaecologists believe that vaginal products for self-administration are valuable, but consider that more pharmaceutical counselling should be provided in view of increasing therapies' compliance and efficacy²⁸⁰. Likewise, in recent years, clinicians and patients' preferences were studied on the different oral and vaginal therapeutic options for vaginal fungal infections, concluding that given the therapeutic efficacy and equivalence of the individual antifungal agents as well as route of administration, treatment selection should be driven by the patient's personal preferences²⁸¹.

II.2.2.2. pH and buffering capacity

The pH of the formulations is an important parameter since it must be compatible with the vaginal pH which is normally 3.5-4.5, but may vary according to specific vaginal conditions (hormonal stimulation, menstrual cycle phase, presence or absence of infections)¹⁵. However, as the pH values of vaginal products do not allow by their own for a complete prediction of their safety, it is more relevant to assess the ability of these formulations to actually change the physiological pH once administered, by other words the pH-buffering capacity. This parameter, especially when determined in VFS, contributes to a better understanding of what will happen *in vivo* regarding pH changes after the formulation is applied in the vagina¹⁵⁹.

Table II. 2 shows the results of pH determinations for the vaginal formulations enrolled in this study. Antifungals in general had low pH, except for Gino-Canesten® that has a pH of 5.89. This fact might be due to poor solubility and stability of clotrimazole in acidic solutions/formulations²⁸², since it is as weak base with a pKa of 6.9²⁸³. Additionally, it should

be considered that fungal infections, which are mainly caused by *Candida spp.*, tend not to affect the normal vaginal pH²⁸⁴. On the other hand, bacterial infections, the most common being vaginal bacteriosis (VB), are characterized by pH increase to 5.00-6.00. Dalacin V® has a pH in the lower limit of the physiologic range (3.54 ± 0.006), which can contribute to reduce the high vaginal pH value present in VB. Since antimicrobial products are intended to an occasional administration (vaginal infections are isolated conditions and usually do not require prolonged therapies) it may be acceptable that they can have pH values out of the physiological range. On the other hand, products as Ovestin® and Blissel®, that are intended to be used for prolonged therapies, should exhibit pH values compatible with the normal vaginal pH for safety issues. In fact, we confirmed that both presented pH values within those considered as physiological (3.92 ± 0.010 and 4.79 ± 0.046 , respectively). Since these two products are prescribed for chronic application this characteristic may help the formulation to have an adequate impact in the vaginal milieu. Moreover, vaginal atrophy in menopause is characterized by vaginal irritation and discomfort, so pH changes due to products application (either acidic or basic) are expected to increase those symptoms. Colpotrophine® has a manufacturer specification for vulvar application other than the vaginal, so its pH is not expected to be comprised within the vaginal range ($\text{pH} = 7.26 \pm 0.040$), but fitting the pH range recommended for skin applications: 4.0 to 7.0²⁸⁵. As shown in **Table II. 2**, after the dilution in VFS for the pH-buffering capacity assay, formulations acquired the simulant pH (4.21), except for Lomexin®, Ovestin®, Blissel®, Colpotrophine® and Replens®, which resulted in pH in statistically different from the control (one-way ANOVA $p < 0.05$). On the other hand, dilution with the NS control, did not affect markedly the formulations pH (data not shown).

To evaluate formulations pH-buffering capacities two different endpoints were considered: the relevant buffering capacity (RBC) and the absolute buffering capacity (ABC). While the relevant buffering capacity represents the ability of a formulation to overcome a pH of 5, meaning that it has left the physiologic interval for the vaginal environment, the absolute buffering capacity represents the ability for a formulation to change 1 pH point from its natural pH¹⁵⁹. **Figure II. 1. A** represents the relevant buffering capacities for all products included in this study. Gino-Canesten®, Colpotrophine® and the NS control are represented in white, since they had initial pH higher than 5 and were titrated with HCl 1 N instead of NaOH 1 N, until $\text{pH} \leq 3.00$. These calculations were performed equally to the other formulations. Although, these two formulations had pH above the physiologic limit, they had low buffering capacities (both RBC and ABC), which means they can easily reach the normal vaginal pH interval.

Table II. 2: pH and osmolality studies of vaginal products included in this study. Results are means \pm SD (standard deviation) (n=3). * represents statistically different from the respective dilution media; ∞ represents statistically different between dilutions with VFS and the undiluted formulation; \ddagger represents statistically different between dilutions with VFS_m and the undiluted formulation (two-way ANOVA, $p < 0.05$).

	Direct pH	Diluted pH (VFS)	Direct osmolality	Diluted osmolality (VFS)	Diluted osmolality (VFS _m)
	pH \pm SD	pH \pm SD	Osmolality \pm SD (mOsmol/kg)	Osmolality \pm SD (mOsmol/kg)	Osmolality \pm SD (mOsmol/kg)
Gino-Canesten®	5.89 \pm 0.07	4.22 \pm 0.01	144 \pm 3	165 \pm 2*	163 \pm 1*
Sertopic®	2.71 \pm 0.01	4.21 \pm 0.01	300 \pm 2	272 \pm 7*	284 \pm 1*
Dermofix®	2.70 \pm 0.02	4.19 \pm 0.03	248 \pm 4	228 \pm 2	240 \pm 2*
Gyno-Pevaryl®	2.74 \pm 0.03	4.07 \pm 0.01	340 \pm 8	264 \pm 3* ∞	266 \pm 2* \ddagger
Lomexin®	3.57 \pm 0.01	4.02 \pm 0.05*	1446 \pm 20	1210 \pm 16* ∞	1253 \pm 18* \ddagger
Gino Travogen®	3.56 \pm 0.04	4.16 \pm 0.00	43 \pm 2	75 \pm 1*	57 \pm 1*
Dalacin V®	3.54 \pm 0.01	4.19 \pm 0.01	1681 \pm 10	1223 \pm 8* ∞	1288 \pm 21* \ddagger
Ovestin®	3.92 \pm 0.01	3.95 \pm 0.03*	3332 \pm 60	938 \pm 7* ∞	1061 \pm 27* \ddagger
Blissel®	4.79 \pm 0.05	4.32 \pm 0.02*	1537 \pm 6	920 \pm 12* ∞	1042 \pm 18* \ddagger
Colpotrophine®	7.26 \pm 0.04	6.76 \pm 0.04*	1723 \pm 20	396 \pm 4* ∞	472 \pm 47* \ddagger
Universal placebo	4.51 \pm 0.04	4.19 \pm 0.02	339 \pm 2	306 \pm 2*	324 \pm 5*
Replens®	2.81 \pm 0.02	4.12 \pm 0.00*	2350 \pm 81	1859 \pm 22*	1903 \pm 17*
Dilution media		4.21 \pm 0.01		212 \pm 1	254 \pm 1

As expected, all products showed higher buffering capacity after being mixed with VFS due to the intrinsic effect of this fluid (lighter grey control, **Figure II. 1. A and B**), when comparing with the capacity of the normal saline solution.

Universal placebo has revealed little buffering capacity even on VFS (similar to the results obtained for this control), probably meaning that its application will not modify the vaginal pH. Dalacin V®, despite having a pH value compatible with the vaginal physiological pH, did not show a favourable RBC on VFS. Although it would be important to retain a low pH in the presence of VB (its main therapeutic indication). Blissel®, unlike Ovestin® labelled for the same therapeutic purpose, had a high RBC, meaning that it is able to maintain the physiological pH in less acidic conditions.

The ABC values for Gino-Canesten®, Sertopic®, Ovestin®, Colpotrophine® and Universal placebo were not statistically different from the control on the NS assay. As for the assays in VFS no differences from the control were found for Sertopic®, Gino Travogen®, Dalacin V®, Ovestin® and Universal Placebo. Overall, buffering capacity is probably related to the presence of acidic polymers, as seen for Blissel®¹⁵⁹. Also, formulations which had pH out of the vaginal physiologic range (Gino-Canesten®, Sertopic®, Dermofix®, Gyno-Pevaryl® and Colpotrophine®), had relatively low capacity to maintain their own pH, which means that by mixing with acidic vaginal fluids they could change to a value closer to the physiological range although not being expected to correct the abnormal vaginal pH associated with the target problem. It is well established that deviations from the normal vaginal pH in the healthy adult (3.5-4.5) are considered as potentially deleterious for the vaginal epithelium¹⁶⁰. Classically, the

acidic pH in the vaginal environment is believed to contribute to the normal physiology, to favour microbiota, and to promote a balanced immune response. Vaginal products should be compatible with vaginal pH and, ideally, maintain it or even help in its reestablishment (e.g., in cases of bacterial vaginosis or post-menopausal vaginal atrophy)¹⁶¹. Even if the consequences of the administration of vaginal formulations presenting undesirable pH is not readily assessable, it is well known that increased vaginal pH is associated with the presence or favours bacterial vaginosis, trichomoniosis or mixed infections¹⁶².

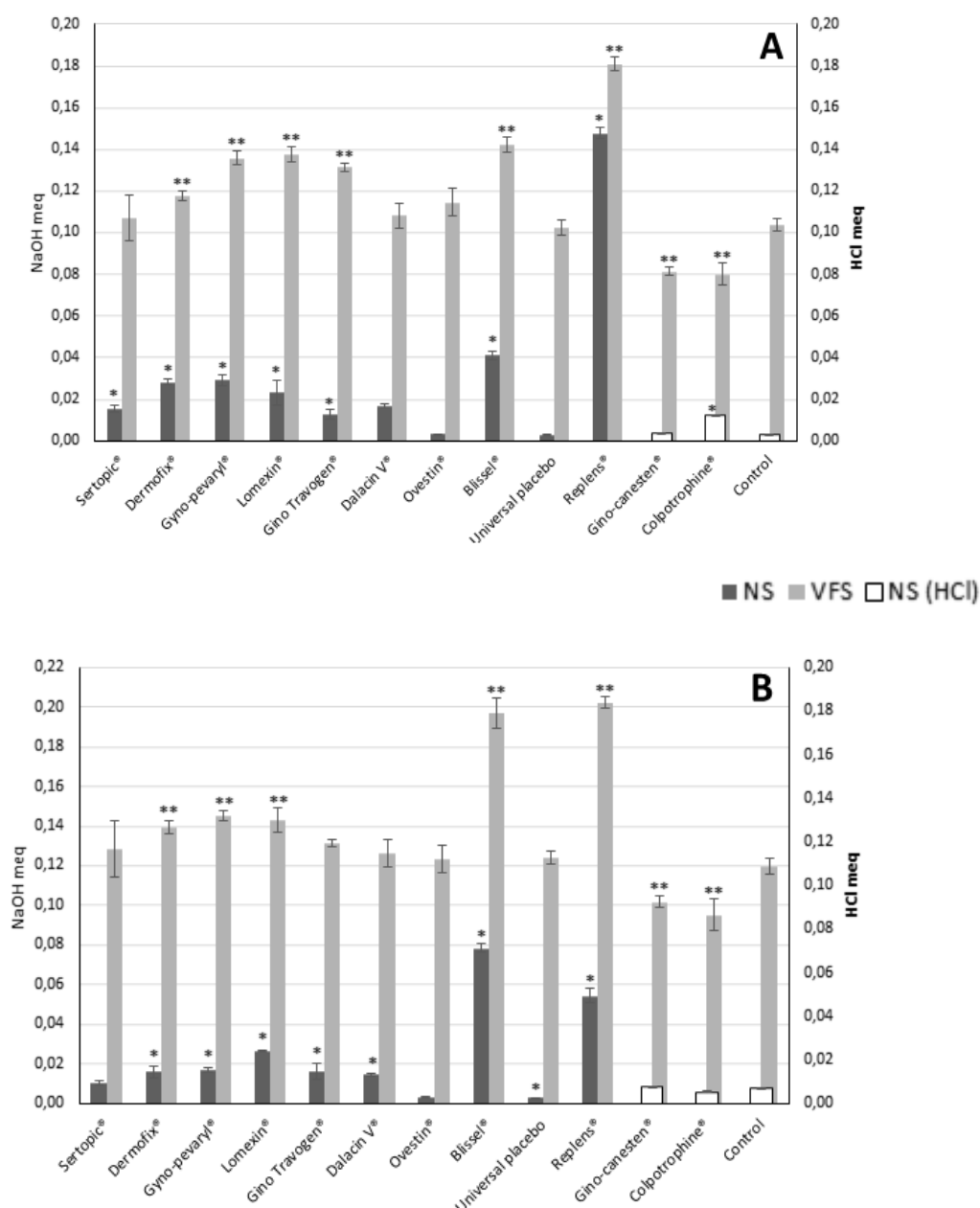


Figure II. 1: (A) Relevant and (B) Absolute pH-buffering capacity expressed as NaOH meq for the vaginal products included in this study. For Gino-Canesten®, Colpotrophine® and Control in NS, the addition was made with HCl, since their pH were higher than 5. Results are means and bars represent standard deviations (n=3). NS=Normal saline; VFS=Vaginal Fluid Simulant; NS (HCl)=Normal saline tritrated with HCl. * represents statistically different from the NS control and ** represents statistically different from the VFS control (one way-ANOVA, $p < 0.05$).

Outcomes of low pH are even less understood, but animal data suggest that values of three or less are generally unacceptable for human use¹⁵⁵. Furthermore, apart from being pH compatible, vaginal products should allow the maintenance of the vaginal acidic environment and oppose pH-raising/decreasing events. Indeed, the use of acid-buffering gels has been proposed for the reestablishment of pH in cases of infection^{66,157} or menopausal atrophy¹⁶³. A study of pH and buffering capacity of diverse marketed vaginal lubricants has already been performed within our workgroup¹⁵⁹ and we concluded that most of the lubricants presented pH and/or osmolality values outside the ranges recommended by the WHO (World Health Organization). This later study made clear the need for further characterization in order to fully understand the potential hazard profile of the vaginal products.

II.2.2.3. Osmolality

Osmolality was determined directly on the formulations, but also after dilution on the correspondent volume of fluid in amounts equal to those expected to be present in the vagina (0.75 mL), using normal VFS and the VFS_m (containing mucin) (Table II. 2). The osmolality of the control VFS (212 mOsmol/Kg) was in accordance to previous results found in the literature²⁸⁶. Although being slightly hyposmolal (considering isosmolal around 300 mOsmol/Kg), this value is similar to the physiological one (260-290 mOsmol/Kg)¹⁶⁵. However, it is expected that a broader interval of osmolality for vaginal administration will be well tolerated, as for ocular delivery, formulations between 260-480 mOsmol/Kg, results in no irritation, although the osmolality of the lacrimal fluid normally ranges between 310-350 mOsm/Kg²⁸⁷.

Osmolality measurements on plain formulations revealed that Lomexin®, Dalacin V®, Ovestin®, Blissel® and Colpotrophine® had higher osmolality than the higher limit recommended by the WHO for lubricants (<1200 mOsmol/kg)¹⁶⁵. On the other hand, Gino-Canesten®, Sertopic®, Dermofix®, Gyno-Pevaryl®, Gino Travogen® and Universal placebo are in accordance to the recommended value, 380 mOsmol/kg. The high osmolalities might be due to the presence of high levels of glycerin and/or propylene glycol in the formulations' composition. Indeed, the WHO recommends that glycerin and propylene glycol concentrations should not exceed 9.9% (w/w) and 8.3% (w/w), respectively¹⁵⁹. However, after mixing on both normal VFS and modified VFS, the final osmolalities were in accordance to this criteria.

For the VFS mixtures, all osmolalities were affected by the dilution, being statistically different from the control (media itself), two-way ANOVA, $p < 0.05$; except for Dermofix®, which already had an osmolality similar to the media. In respect to the mixtures on VFS_m only Sertopic®, Dermofix® and Gyno-Pevaryl® were statistically affected by the dilution (two-way ANOVA, $p < 0.05$). When comparing VFS diluted with undiluted formulations' osmolalities, there is a significant difference in almost all formulations except for Gino-Canesten®, Sertopic®, Dermofix®, Gino Travogen® and Universal Placebo. This behaviour was already expected, since they were mixed with a fluid with similar tonicity (VFS=212 mOsmol/kg), not affecting the final dilution. While comparing undiluted formulations with the diluted ones in VFS_m there are few

differences. The resulting osmolality was only statistically different for Gyno-Pevaryl®, Lomexin®, Dalacin V®, Ovestin®, Blissel® and Colpotrophine®. These results show that, while there are few statistical differences between diluted and undiluted formulations whatever the dilution media is, it might be of special interest to consider the evaluation of osmolality in a physiological dilution. This dilution clearly indicates the potential irritation that might be associated with the product, which is not deducted when measurements are made directly on the formulation. So, for future determinations a complete osmolality assessment should not only comprise the direct measurement but also the dilution in the VFS, the most used by research groups. The osmolality of the resulting dilution has the capacity to early predict the *in vivo* formulation behaviour, representing an initial strategy for safety foresee.

WHO in collaboration with the United Nations Population Fund (UNFPA) and Family Health International (FHI360), recently issued an “Advisory Note” on the technical requirements of vaginal lubricants, namely when used in addition to condoms. Osmolality has been highlighted, and specific recommendations have been proposed: values of 380 mOsmol/kg or lower are desirable (hypo- and isosmolal), but values as high as 1200 mOsmol/kg have been considered acceptable on an interim basis¹⁶⁵. Available pre-clinical and clinical data support that hyperosmolal vaginal products may be related to safety issues¹⁶⁶, as well as detrimental effects on sperm motility, viability and chromatin quality^{167,168}. Vaginal products’ safety goes beyond chemical toxicity to include physical parameters such as osmolality¹⁶⁴. If a formulation is excessively hyperosmotic there is the potential to cause irritation leading to an inflammatory response. Each dissolved ingredient in a topical formulation contributes to the final product osmolality, so the effect of the overall composition of the formulation must be considered in addition to the impact of each individual ingredient. In general, the loss of cell viability is possibly correlated to the gels’ osmolality; the higher the solute concentration, the greater the dilution that is needed to maintain viability^{159,164}.

On the other hand, hypotonic formulations can conduct to increased fluid absorption, leading to higher permeation rates of drugs and nanoparticles through the vaginal epithelial surface²⁸⁸. Hypotonic formulations toxic effects (especially irritant) in vaginal administration have not been largely investigated. Ensign *et al.* hypothesized the administration of hypotonic solutions and found that hypotonic formulations markedly increased the rate at which small molecule drugs and muco-inert nanoparticles reached the vaginal epithelial *in vivo* in mice. Furthermore, using a mouse model of vaginal genital herpes (HSV-2) infection, these researchers found that hypotonic delivery of free drug led to improved immediate protection, however, diminishing longer-term protection.

II.2.2.4. Firmness and Adhesiveness

Textural characteristics of vaginal formulations are important not only in view of effectivity, but also in what concerns to patient compliance²⁸⁹. Conventional semisolids for vaginal administration are reported to suffer from relatively low patient acceptability and poor vaginal retention and so, the development of new vaginal semisolids requires a fundamental

understanding of their rheological and textural properties within the vaginal cavity²⁹⁰. Several works have reported the use of firmness and adhesiveness determinations in view of texture characterization of vaginal products^{169,170}. Also, the measurement of the work of syringeability has been applied for a vaginal applicator model in order to determine expelling capacity of applicators²⁹⁰.

Firmness and adhesiveness were determined as physic-mechanical characteristics of the formulations and were studied in view of a correlation between these two parameters. However, as shown by **Table II. 3**, it is not evident that the higher the adhesiveness the lower/higher the firmness. In fact, these two characteristics do not have any correlation neither on the Spearman non-parametric test ($r=0.2657$) nor on the Pearson parametric test ($r=0.0939$), with confidence level of 95%. Higher adhesiveness conducted to low firmness, such as the case of Universal placebo and even Blissel®, but not statistically supported. These two formulations are the only ones with a polymeric composition and this behaviour may be due to this type of excipients (hydroxyethylcellulose and a combination of polycarbophil/carbomer, respectively). Antifungals had almost the same textural behaviour: medium adhesiveness (0.400-0.600 N.mm, approximately) and medium to high firmness (0.070-0.300 N, approximately). Dalacin V®, the only antibacterial product enrolled in the study, presented similar results to the antifungals. This might mean that this texture is adequate to short-term therapies, and also easy to filling-in the applicator and posterior administration. Actually, several excipients are common to this group of formulations (antimicrobials), such as, liquid paraffin, cetyl palmitate, propylene glycol and cetostearyl alcohol, although not being known their quantitative composition. Topical oestrogens had different textures among them. While Blissel® showed to be averagely adhesive, it is the less firm of all formulations, meaning that it could have a good spreadability over the vaginal epithelium. On the other hand, Ovestin® despite being a little more adhesive, it is also more firm and this could lead to lower spreadability over epithelium. Although more adhesive in the mechanic test, Ovestin® was less bioadhesive than Blissel®, when a physiologic feature was endorsed. This might be due to the combination of polycarbophil/carbomer on Blissel®, a recognized derivate highly adhesive acrylic acid⁹³.

Formulation characteristics, including viscosity, elasticity, and rheology, are the most important factors in the development and final behaviour of semisolid formulations. Also, temperature and site of application are of extreme importance for formulations spreadability²⁹¹. To assess the spreadability of a topical or a mucosal semisolid preparation, the important factors to consider include hardness or firmness of the formulation, the rate and time of shear produced upon smearing, and the temperature of the target site. The rate of spreading also depends on the viscosity of the formulation, the rate of evaporation of the solvent, and the rate of increase in viscosity with concentration that results from evaporation²⁹².

Table II. 3: Mechanical (adhesiveness (N.mm) and firmness(N)) and bioadhesive parameters (work of adhesion (N.mm), peak force-adhesiveness (N) and debonding distance (mm)) determined for the products in study. Results are means \pm SD (standard deviation) (n=3). * represents statistically different from the control (one-way ANOVA, $p < 0.05$).

Formulations	Adhesiveness (N.mm) \pm SD	Firmness (N) \pm SD	Work of Adhesion (N.mm) \pm SD	Peak Force-Adhesiveness (N) \pm SD	Debonding Distance (mm) \pm SD
Gino-Canesten®	0.531 \pm 0.050	0.206 \pm 0.007	0.022 \pm 0.001	0.033 \pm 0.015	2.160 \pm 0.044
Sertopic®	0.401 \pm 0.071	0.123 \pm 0.010	0.019 \pm 0.006	0.020 \pm 0.000	2.337 \pm 0.452
Dermofix®	0.438 \pm 0.070	0.131 \pm 0.008	0.028 \pm 0.008	0.020 \pm 0.000	3.167 \pm 1.024
Gyno-Pevaryl®	0.500 \pm 0.032	0.073 \pm 0.001	0.061 \pm 0.008*	0.060 \pm 0.020	2.613 \pm 0.431
Lomexin®	0.563 \pm 0.041	0.260 \pm 0.021	0.070 \pm 0.023*	0.117 \pm 0.050	2.547 \pm 0.573
Gino Travogen®	0.407 \pm 0.047	0.134 \pm 0.007	0.032 \pm 0.008	0.037 \pm 0.006	2.280 \pm 0.137
Dalacin V®	0.364 \pm 0.009	0.124 \pm 0.006	0.020 \pm 0.004	0.033 \pm 0.006	2.070 \pm 0.140
Ovestin®	0.827 \pm 0.055	0.204 \pm 0.009	0.021 \pm 0.003	0.030 \pm 0.017	2.027 \pm 0.387
Blissel®	0.638 \pm 0.022	0.077 \pm 0.002	0.041 \pm 0.001*	0.057 \pm 0.029	2.087 \pm 0.137
Colpotrophine®	0.390 \pm 0.011	0.092 \pm 0.002	0.026 \pm 0.011	0.037 \pm 0.015	2.117 \pm 0.156
Universal placebo	1.198 \pm 0.008	0.078 \pm 0.001	0.110 \pm 0.021*	0.140 \pm 0.069	2.930 \pm 0.543
Replens®	0.181 \pm 0.026	0.065 \pm 0.014	0.047 \pm 0.002*	0.070 \pm 0.017	2.147 \pm 0.199
Control			0.015 \pm 0.002	0.027 \pm 0.012	2.010 \pm 0.111

II.2.2.5. Bioadhesion

Textural analysis is essential for product characterization. However, studies on formulation mechanical behaviour on a biologic perspective should also be addressed. Bioadhesion represents the ability of a formulation to adhere to a biological surface, in this case, the vaginal epithelium.

The one-way ANOVA ($p < 0.05$) statistics determined that, regarding Work of Adhesion (herein considered as bioadhesion), only Gyno-Pevaryl®, Lomexin®, Blissel®, Replens® and Universal placebo were different from the control (performed without any formulation) (Table II. 3). The Work of Adhesion was compared with the pure textural parameters and it was found a moderate to strong uphill positive linear correlation with Adhesiveness (Pearson, CI 95%, $r=0.6233$). This can represent a valuable information, since it means that bioadhesion could be predicted by adhesiveness, a test that does not require the use of biological surrogates and can easily be performed in earlier stages of product development. These results were obtained with an experimental setup that considers the physiological temperature in order to reflect the formulations behaviour in a more physiological condition.

Mucoadhesion (herein referred as bioadhesion since the vaginal epithelium is not considered as a mucosa) represents an attractive interaction that involves a pharmaceutical dosage form and either secreted mucus or a mucosal/epithelial membrane¹⁷². Bioadhesive properties allow better contact of the formulation with the vaginal surface and longer residence times. In most cases, bioadhesion is imparted to a formulation by the employment of polymeric excipients. The mechanisms of bioadhesion involve, firstly, a contact stage, hydration, wetting and spreading (which are the most important steps), and subsequently a consolidation stage, that involves the strengthening of polymer-mucin joint, thanks to the inter-penetration of the polymer chains into the mucus layer and the occurrence of polymer-mucin bonding (mainly

weak van der Waals and hydrogen bonds or electrostatic interactions)^{172,173}. Maximum force of detachment (F_{\max}) (directly measured) and the work of adhesion (W_{ad}) (calculated as the area under the curve force vs displacement) were the parameters used to evaluate the bioadhesive potential. The reliability of a tensile method is strictly dependent on the failure in the interfacial (mucin/polymer) region: in particular it is difficult to distinguish where the failure of the bioadhesive joint occurs and if the cohesive nature of the sample (failure within the polymer layer) or the strengthening of the mucus layer (failure within the mucus layer) plays the major role¹⁷³.

The natural mild slope of the vaginal canal, in association with its self-cleansing mechanisms (e.g. fluid transudation) and possible mechanical stress (e.g. during penile penetration), contributes to the expulsion of products placed in the vagina. Another important issue impacting the bioadhesion phenomenon is related to the variability of the vaginal fluid with the menstrual cycle and hygiene practices (e.g. douching). Vaginal fluid can undergo either quantitative or qualitative changes, namely in pH, mucin content and rheology. These factors influence the interaction of bioadhesive formulations with mucin, namely by changing the conformation and properties of the network formed by mucin within the vaginal fluid¹⁷⁴.

Bioadhesive dosage forms or delivery systems can contribute to prolonged *in situ* residence, resulting in advantageous features such as fewer applications needed, reduced vaginal leakage, and intimate contact between drugs and the epithelial tissue. Different dosage forms have been formulated as bioadhesive namely tablets, suppositories, creams, and gels⁹⁸. Indeed, one of the first enthusiast reports on a specific bioadhesive vaginal gel dates back to the 90s by Robinson and Bologna¹⁷⁵. The bioadhesive properties of the proposed gel, currently commercialized as Replens® (Lil' Drug Store Products, Inc.), were attributed to the inclusion of an acrylate polymer, polycarbophil (1-3%). Since then, these polymers have been used as classical bioadhesive and gelling agents for the formulation of various commercially available vaginal gels¹³⁶. After that, Garg *et al.* proposed a new mucoadhesive gel, ACIDFORM which was shown to present enhanced *in vitro* mucoadhesive properties when compared to various commercial gels¹⁵⁷. It is composed by acidic substances (lactic acid, citric acid and potassium bitartrate), a preservative (benzoic acid), gelling agents (alginic acid and xanthan gum), a humectant (glycerin), sodium hydroxide and water²⁹³.

The common strategy for increasing bioadhesion of vaginal dosage forms, especially among research published papers, has been to use well known bioadhesive polymers such as polyacrylates, chitosans, cellulose derivatives, hyaluronic acid and derivatives, pectin, starch, and several natural gums, among others⁹³. Acidic polymers such as polyacrylates present the additional feature of contributing for the acidic pH-buffering of the vaginal milieu within the desirable normal range, and thus potentially contributing to a healthy vagina¹⁷⁶. Regarding chitosan, its intrinsic ability to interact with intercellular tight junctions and to inhibit proteolytic enzymes provides additional mechanisms for promoting the vaginal absorption and peptide/protein protection from degradation, respectively⁷⁶. In recent years, thiolated polymers have also been tested for designing vaginal dosage forms with improved bioadhesive

performance when compared to their non-thiolated counterparts¹⁷⁸. Even if substantial success has been achieved, much of the rationale behind the choice of bioadhesive polymers for vaginal formulation derives from studies intended to evaluate these excipients for use in other mucosal routes¹⁷⁹. Also, a formulation to deliver controlled doses of progesterone based on cyclomethicone was prepared as a silicone-water emulsion, with great potential to be bioadhesive, even after dilution on VFS²⁹⁴. The bioadhesive potential of polymers and derived dosage forms is also dependent on the specificities of the mucosal environment, and its evaluation should take this into account. For instances, *in vitro* experimental settings relevant to the vaginal physiology, namely pH values, have been shown to significantly influence the bioadhesive performance of vaginal semisolid formulations¹⁸⁰. This need for mimicking the vaginal environment led to the development of different specific *in vitro/ex vivo* experimental protocols for evaluating the bioadhesive potential of vaginal dosage forms¹⁷³. Proposed techniques generally involve measuring the forces involved in the detachment of a formulation from a model synthetic or natural mucosa. Alongside, imaging techniques have been used to evaluate bioadhesion *in vivo*²⁰. Several methodologies have been applied to determine bioadhesion¹⁷³. However, the one herein described gave reproducible results, skipping their major difficulty when working with biological surrogates. Furthermore, our work reflects the usage of a standardized equipment and method. Finally, the assembly of this methodology conducted to the establishment of a correlation between parameters determined in the same equipment (adhesiveness and bioadhesion).

II.2.2.6. Viscosity

Viscosity, i.e. rheological properties are of great interest given the effect these may have on drug release properties and passive outflow between epithelial surfaces. Moreover, rheological properties are of primary interest because they have been shown to largely govern the ease of application and dispersion of semisolids²⁹⁰, which will obviously influence the ability of the formulation to coat the vaginal cavity therefore providing efficacy.

Thixotropic profiles were outlined at room temperature (25°C) directly over the formulations in study (**Figure II. 2**). Sertopic®, Dermofix®, Blissel® and Universal placebo had lower viscosities, and consequently lower shear rates were necessary to obtain an acceptable torque.

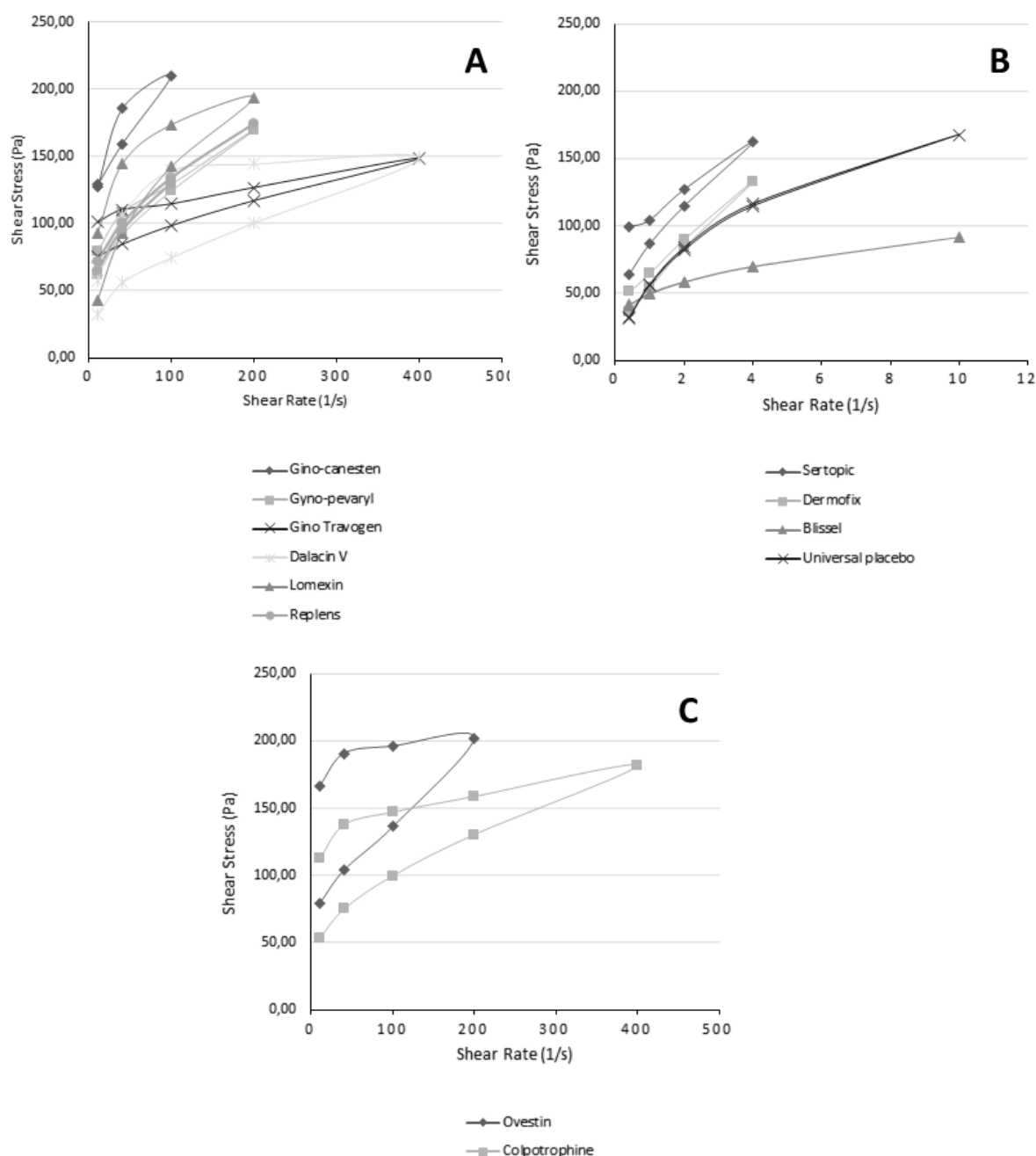


Figure II. 2: Viscosity represented as Shear Stress (Pa) vs Shear Rate (Pa) demonstrating thixotropic behaviour for (A) antimicrobials and Replens® gel; (B) topical oestrogens; and, (C) Low viscosity formulations. Results represent the mean of 3 independent determinations.

Concerning **Figure II.2.A** it is clear that all antimicrobials have a thixotropic textural behaviour (Non-Newtonian - pseudo plastic), which is not so marked on the Replens® formulation. Sertopic® and Dermofix®, although having lower viscosities show also this time-dependent behaviour. Thixotropic materials become more fluid as shear rates decreases, short after an increasing shear rate testing. On the contrary, Blissel® and Universal placebo showed not to be affected by time-dependent viscosity determinations (**Figure II. 2.B**).

Viscosities were also determined after mixture with the VFS, at 25°C, 37°C and considering the administration in an *ex vivo* model (**Figure II. 3**). These dilutions and temperature

adaptations can better mimic the rheology adopted after application by the vaginal products. In general, viscosity was clearly lower when compared to the plain formulations. Also, the thixotropic phenomenon for most of formulations was less marked (data not shown). Differences were statistically significant (two-way ANOVA, Multiple Comparisons, $p < 0.05$) for all dilutions at 37°C compared with dilutions at 25°C except for Dermofix® and Blissel®. And for all dilutions at 25°C compared with undiluted formulations measured at the same temperature. Viscosity was highly dependent on temperature^{26,287}, as expected. However, the variation observed, was not proportional nor similar among all formulations. Each formulation had its own behaviour, driven by their composition. This could mean that measurements directly made on formulations at room temperature do not represent the viscosity acquired after administration. Furthermore, measurements upon dilution with VFS at room temperature (25°C) would still be significantly different from viscosities obtained at physiological temperature. Considering the test using the *ex vivo* porcine vagina, it was showed that also this model could be valuable, not only because it mimics the *in vivo* administration, but also it brought different results from the dilution at 37°C. For almost all formulations the viscosity was higher in this test, except for Gino-Canesten® and Sertopic®, when comparing to the formulations under dilution at 37°C. In fact, this was an unforeseen result, since the effect of rotation movement was expected to decrease the overall viscosity of the sample with the VFS and the vaginal environment. However, these results are quite satisfying in what concerns to comfort and leakages issues. The fact that the formulations can adopt, after administration, a higher viscosity, could circumvent these problems. Furthermore, these results could highlight the need to establish the model herein presented, to better predict the formulations' rheology after administration.

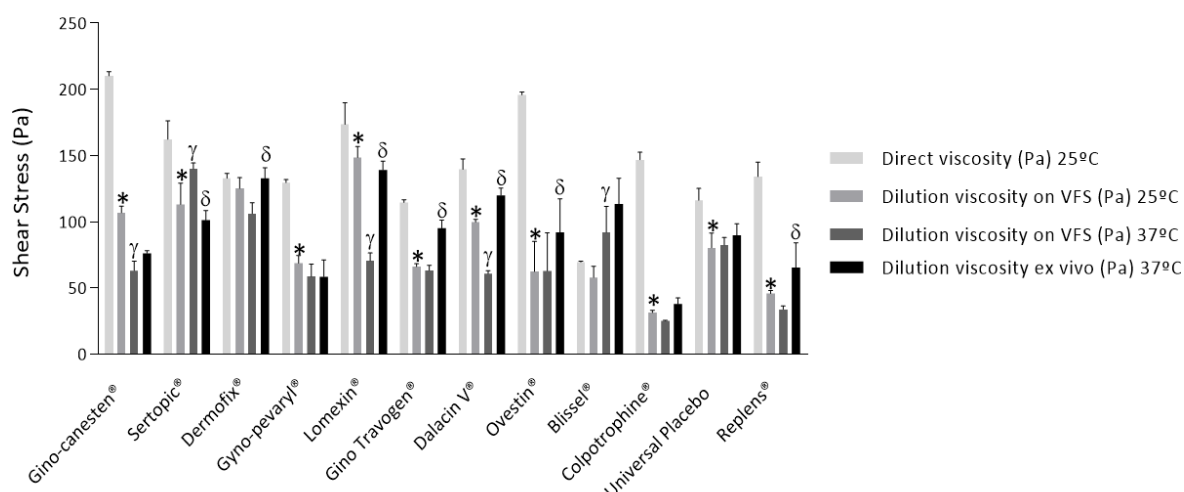


Figure II. 3: Viscosity (as Shear stress (Pa)) comparisons for direct measurements and diluted measurements at temperatures of 25°C and 37°C. Within formulations the same Shear rate (1/s) was considered. Results correspond to the mean and bars to the standard deviation of 3 determinations. Viscosity comparisons were assessed only within formulation. * represents statistically different from direct viscosity; γ represents statistically different from the dilution at 25°C and δ represents statistically different from the dilution at 37°C (two way-ANOVA, $p < 0.05$, Tukey's multiple comparisons test).

Aka-Any-Grah *et al.*, have also reported major differences among formulations developed with hydroxypropylmethyl cellulose (HPMC) and pluronics F127/F68, especially on the gelling temperature (before and after dilution in VFS), rheological properties and even *ex vivo* adhesion¹⁸². This data suggests that approximations to vaginal physiological conditions are determinant to foresee *in vivo* rheology of the formulations after administration¹⁸¹. Viscosity can dictate the ability of the formulation to disperse *in vivo*, as well as the residence time in the genitourinary tract of these formulations, hence it is decreased owing to the self-cleansing action of the vagina and the dilution with vaginal fluids, and even environmental temperature¹⁸².

Katz *et al.* disclosed the rheological properties of Advantage-S® and Replens® at body and room temperature over a range of physiologically relevant shear rates. It was found that their rheological behaviour was different among temperatures and miscibility with vaginal fluid simulant was also affected²⁹⁵.

Lai *et al.* (2008) studied the effect of small dilutions (10-30%) in vaginal fluid and semen simulants on K-Y Jelly®, Replens® and Carraguard®, using a cone-plate rheometer, and data was fitted to power-law, Carreau, or Herschel-Bulkley models. Rheological parameters from these fits were input to models of coating flow due squeezing, and the simulated area coated output from these models was used to compare the responses of the different formulations to the two diluents for varying degrees of dilution. There were differences in the responses of the three materials to dilution; even small dilutions altered the rank order of vaginal coating rates compared to the undiluted formulations²⁸⁶.

Later on, Henderson *et al.* (2007) used an optical imaging technique to compare human intravaginal coating distributions of Conceptrol® and Advantage®. It was concluded that the results were consistent with those predicted through mechanistic coating theory, using gel rheological data as input²⁹⁶. Furthermore, in 2008, Mauck *et al.* studied the vaginal distribution of Replens® and K-Y Jelly® *in vivo* in women. Time, ambulation, parity and body mass index were factors considered for vaginal spreading. Imaging was achieved by magnetic resonance imaging, gamma scintigraphy and with a fiberoptic probe. Results showed that the initial application of the gel resulted in two thirds of maximum coverage possible, both in linear extent along the vaginal axis and in surface area covered. Over the next 45 min, spreading increased to about three quarters of the maximum possible. Ambulation generally increased linear spreading. Effects of parity and body mass index were similar on most measures of gel spreading, with nulligravid women tending toward greater spread than parous women and women of high body mass, usually showing somewhat greater spread than women of normal weight. Differences between the two gels were not seen when all conditions of application were considered together²⁹⁷.

Recently, Katz *et al.* described the fundamental principles of mass transport, highlighting the diffusion and convection of drugs in the vaginal environment. Several mathematical predictive models can be used to this, although having some variability when compared to *in vivo* behaviour. These models can illustrate drug concentration distribution (pharmacokinetics)

and effectiveness (pharmacodynamics). Modelling can be used to compare vaginal drug distributions after different gel dosage regimens, comparing the effect of vaginal fluid and the consequences of changes in gel viscosity due to aging. It could also be helpful in comparing drug distribution after the application of different dosage forms. Ultimately, the modelling approach is used to compare vaginal drug distribution across species with differing vaginal dimensions²⁹⁸.

Anwar *et al.* have investigated the interplay between vaginal tissue elasticity and the yield-stress of non-Newtonian fluids during a microbicide deployment. Yield stress is the applied stress one must exceed in order to make a structured fluid flow, being the “force” implied on the formulation at the beginning of a viscosity determination. Within this research work this group has developed a mathematical model of tissue deformation driven by spreading of microbicidal gels based on thin film lubrication approximation and demonstrated the effect of tissue elasticity and fluid yield-stress on the spreading dynamics. It was concluded that both elasticity of tissue and yield-stress rheology of gel are strong determinants for the coating behaviour²⁹⁹.

In another study, Kieweg *et al.* performed experimental and numerical studies on microbicidal gel deployment under constant squeezing force and concluded that squeezing force, gel consistency, shear-thinning behaviour and yield stress are strong determinants of the coating performance of gels³⁰⁰⁻³⁰². Szeri *et al.* developed a mathematical model involving wall elasticity to demonstrate the effect of compliant vaginal wall on the deployment of new formulations³⁰³.

Vaginal gels should be highly elastic, even after dilution, as these properties govern drug release and leakage. Furthermore, pseudoplasticity would offer stress-induced viscosity depression and hence ensure ease of application³⁰⁴. This is difficult to achieve using single polymer gels. Therefore, gels offering greater clinical promise may be achieved through the combination of mucoadhesive and gel structuring polymers within a binary or higher polymer platform^{305,306}.

Omar *et al.* in 2014 proposed an universal vaginal applicator able to homogeneously distribute the formulations over the entire vaginal and cervical epithelia. The internal distribution was investigated using pelvic magnetic resonance imaging (MRI) in a group of women which used six different vaginal gels and creams. Comparisons were made against the conventional applicators. The universal applicator showed to have good potential to reach uniform coverage of vagina and cervix, and enhance women protection against sexually transmitted diseases¹⁰⁴.

Despite of comfort and administration issues, semisolid formulations distribution is directly related to therapeutic efficacy. The use of a gel with a low viscosity would facilitate spreading and hence contact with the vaginal epithelium. However, a low viscosity gel would be expected to have a limited residence time due to the inability of the gel to resist dilution from vaginal fluids and semen, except if it can exhibit a trigger gelling behaviour^{98,173}. Moreover, a low viscosity gel would be unable to “absorb” *in vivo* stresses without causing destruction of

polymer gel entanglements and thus would be expected to leak rapidly. Conversely, a highly elastic gel would offer greater resistance to dilution and to *in vivo* stresses; however, application and intravaginal spreading would be limited. Therefore, optimal clinical performance may only be achieved when the elastic-viscous balance is carefully controlled³⁰⁴. The performance of a vaginal gel can be evaluated with respect to a number of properties, including spreadability, coating and retention by using *in vitro*, *ex vivo* and *in vivo* methodologies³⁰⁷.

II.2.3. Conclusions

In view of designing new formulations for vaginal administration driven by safety and efficacy rationals; biological criteria should be addressed from the early steps of development to accelerate the whole process. The present study showed that a great number of commercial therapeutic vaginal formulations currently used did not present ideal technological characteristics when tested under a physiological perspective. Furthermore, pH buffering capacity, osmolality and viscosity determined using these methodological adaptations were considered focal points to be addressed during products' development. While great effort has been made in the development of innovative vaginal gels in the field of microbicides, antimicrobials and oestrogens formulations have not been the focus of attention in the last years. Nevertheless, they represent the most widely and frequently prescribed all over the world for acute and chronic conditions, respectively. Polymer-based strategies could be applied to re-formulate products already marketed in order to overcome problems of leakage and discomfort, and improve efficacy. The adaptation of these formulations and the use of the methodologic adaptations proposed in this work may optimize cost-efficiency of new and renewed formulations development by predicting efficacy and safety profiles at early stages of product development.

CHAPTER III

PRECLINICAL SAFETY CHARACTERIZATION: CELL-BASED AND ORGAN-BASED (*IN VITRO* AND *EX VIVO*) TESTING

The content of this chapter is partially published in:

Machado RM, Palmeira-de-Oliveira A, Breitenfeld L, Martinez-de-Oliveira J, Palmeira-de-Oliveira R, *“Optimization and application of in vitro and x vivo models for vaginal semisolids safety evaluation”*.

(submitted manuscript)

Palmeira-de-Oliveira R, **Machado RM**, Palmeira-de-Oliveira A, Martinez-de-Oliveira J, *“Testing vaginal irritation with the HET-CAM assay: an in vitro alternative to the in vivo method”*

(accepted manuscript)

III.1. GENERAL CONSIDERATIONS

Concerning all the particularities of vagina dosage forms added to women's preference and acceptability patterns for vaginal semisolid formulations^{22,23}, a safety- and suitability-driven study is expected to conduct to a better characterization of the already marketed products (which are considered safe based on clinical trials and current use) in view of the development of new products.

Over the last decades, efforts have been made to conduct investigation to a level of high comprehension of vaginal epithelium mechanisms⁶. The understanding and application of several techniques to predict drug toxicity through the vaginal epithelium contributes to the successful selection of drugs and appropriate formulations in the early stages of development⁴². Thus, it is of great importance to implement accurate and reproducible methods to predict drug toxicity. The *in vitro* and *ex vivo* approaches should be privileged, and preferably an optimal *in vitro/in vivo* correlation should be established¹⁴².

Despite being an internal cavity, the vaginal epithelium is constantly exposed to potential pathogenic microbes, microflora, chemicals, and hormonal changes. As minor injuries can occur either mechanically or chemically after products usage/administration, further damage or infection may be promoted¹⁵⁹. Vaginal pharmaceutical, cosmetic and personal care products can occasionally induce undesirable local or systemic side-effects. Still, the standard method for assessing vaginal mucosal irritation is the *in vivo* rabbit vaginal irritation test^{42,183,196}. Nonetheless, the current mind-set in toxicology is to use alternative *in vitro* methods that reduce, or, even better, replace the use of animals (3Rs), with a refined profile that modulate and predict human, not animal, responses^{42,199}. This approach is of particular importance in the field of personal care and cosmetic industries since they have to comply with the European legislation, such as the 7th Amendment to the EU Cosmetics Directive that does not allow the marketing of cosmetic products if they, or their ingredients, have been tested for irritation responses in animals³⁰⁸. Safety concerns are currently spotted right through the early stages of development, especially in what concerns to microbicides formulations. This preoccupation was even more highlighted after the unexpected findings of nonoxynol-9 (N-9) in clinical trials³⁰⁹, stressing the need for more appropriate *in vitro* assays to predict *in vivo* safety issues.

Cytotoxicity assays constitute a gold standard of *in vitro* preclinical evaluations of chemicals in cultured cells. The MTT reduction assay is one of the most common applied cytotoxicity assays. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a water soluble yellow tetrazolium salt, which is converted to an insoluble purple compound (formazan) due to cleavage, within the mitochondria, of the tetrazolium ring by, mainly succinate dehydrogenase. Formazan does not permeate cell membranes, and consequently it accumulates in metabolic viable cells. The MTT assay was tested for its validity in various cell lines³¹⁰ and has already been applied to uterine¹⁸⁵, cervical¹⁰⁰ and vaginal³¹¹ derived cells. Further modifications of the initial protocol described by Mosmann³¹⁰ were proposed^{312,313} in order to improve the repeatability and the sensitivity of the assay.

The Neutral Red Uptake (NRU) assay is also a quantitative colorimetric method. It is based on the ability of viable cells to incorporate and bind (uptake) the red dye within the

lysosomes¹⁹³⁻¹⁹⁵. Although they are used extensively as convenient and rapid measurements of cell viability, all these methods should be carried out with some caution. In fact, an increase in NR uptake can be induced by lysosomal swelling agents such as weak alkaline substances and osmotic swelling agents. Regarding the MTT assay, some reducing agents and respiratory chain inhibitors could affect the MTT formazan formation. The MTT assay was also found to be significantly influenced by a number of parameters such as medium pH, D-glucose concentration in culture media, and cellular concentration of pyridine nucleotides³¹⁴. Additionally, the NRU assay presents some advantages over the MTT assay. The former procedure is more sensitive and readily quantifiable. It is at least two times cheaper, presents less interferences, and does not use an unstable tetrazolium salt¹⁹⁵. As a general remark, these assays have, in common with other cell culture procedures, limitations concerning the substance chemical characteristics, say to be volatile, unstable or explosive in water, only very partially soluble, colourant and chemical variably incompatible with the test substance^{191,195}.

In vitro cellular and *ex vivo* tissue models, although being widely applied for toxicity testing, are still far from the complexity of an organ-based model. Furthermore, only specific biomarkers used in these methods, such as interleukins, could provide a predictive response of the irritant potential of vaginal drugs/products. Additionally, these models require the use of appropriate products dilutions due to models' sensitivity. That is why researchers and regulatory agencies are working together to find, optimize and validate the most complex *in vitro* models (organ-based), with the higher similitude with the *in vivo* models.

Topical toxicity has been a main topic within European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM). Having also a regulatory responsibility, the EU and associated laboratories, have been working on developing and validating toxicity test methods for eye irritation, phototoxicity, skin corrosion, irritation and sensitisation³¹⁵. Furthermore, several *in vitro* Test Guidelines are already provided or being developed by the Organisation for Economic Co-operation and Development (OECD)³¹⁶. *In vitro* studies have been valued and recommended as a first screening for toxicity evaluation, in detriment to *in vivo* models.

The assessment of irritability and toxicity of products for vaginal administration does not yet include *in vitro* methods validated and recognized by both the referred authorities. Currently, there are numerous medicines and medical devices marketed or being in the development phase for vaginal administration^{2,4,5,7}. The parallelism between sensitivity to eye and vaginal irritants is addressed in the ISO 10993 guideline since eye irritant products are included in the exclusion criteria for vaginal irritation test, being considered irritant to the vagina³¹⁷. As a general assumption, maximum effectiveness with the least of adverse effects is always desired for vaginal formulations. Since these products are mostly for self-administration and should conceive maximum comfort both at the moment of application and during the time of usage, effects such as local irritation become especially important to be evaluated. Although various techniques for the characterization of vaginal formulations have been described on the literature^{99,153,157,159}, most have been applied only to developing formulations with no

correlation with *in vivo* results. One of the methods most used to assess vaginal irritation is performed *in vivo* on rabbits (RVI)^{316,318}, which must be generally avoided in pursuance of the 3Rs policy (Replacement, Reduction, Refinement) and is even prohibited concerning the marketing of cosmetic products³⁰⁸.

The HET-CAM assay is one of the alternatives presented by ECVAM for the *in vitro* study of ocular irritability^{184,319}. The HET-CAM assay outcome consists on an Irritation Score (IS) that is a value calculated by different analysis methods (A and B), which is used to classify the irritancy potential of a test substance. The IS (A) analysis method takes into account the observation of endpoints at specified time points after application of the test substance (0.5, 2, and 5 min post exposure). At the time points, the presence of an endpoint is determined and a score assigned, in case it is present. The scores are calculated to yield an overall irritation score. Instead, when applying the IS (B) analysis method, the endpoints are monitored over the entire observation period after applying the test substance (typically 5 minutes). The time (in seconds) when an endpoint develops is registered and these values are used to yield an overall irritation score using a mathematical formula³²⁰.

The HET-CAM assay is already widely applied to ocular application products, yet its possible application for the testing of vaginal formulations is an innovative proposal.

III1.1. Objectives

The main aim of the present chapter is to perform a preclinical safety characterization based on cellular and organotypic models (*in vitro* and *ex vivo*). Considering this, it is pretended to:

- Apply the MTT and NRU assay to cervical, uterine and vaginal cell lines in compliance with ISO/EN 10993-5 guide for *in vitro* evaluation of medical devices, to infer cytotoxicity of final formulations dilution through these *in vitro* models;
- Optimize, develop and apply an *ex vivo* strategy to determine tissue viability after the application of vaginal semisolids using the MTT reduction assay and histological analysis and comparing the obtained results with the *in vitro* model;
- Translate the HET-CAM assay as an *in vitro* organotypic alternative method to the vaginal field by testing vaginal semisolid marketed medicines and lubricants concerning their irritation potential under two scoring categories, IS (A) and IS (B).

III.2. EXPERIMENTAL

III.2.1. Materials and Methods

III.2.1.1. Tested Formulations

Ten different semisolid vaginal products that are commercially available in Europe and the USA were included in this study: Gino-Canesten® (Bayer, Portugal), Sertopic® (Ferrer, Portugal), Dermofix® (Azevedos Laboratories, Portugal), Gyno-Pevaryl® (Johnson & Johnson, Portugal), Lomexin® (Jaba Recordati, Portugal), Gino Travogen® (Bayer, Portugal), Dalacin V® (Pfizer Laboratories, Portugal), Ovestin® (Aspen Pharma, Portugal), Blissel® (ITF Medivida, Portugal), Colpotrophine® (Teva Pharma, Portugal). Replens® (Laboratoires Majorelle, France)^{14,93,158,166,173,272} and Universal Placebo^{99,233,321,322} were used as reference products, since their toxicity profile is largely described on the literature. Universal Placebo was prepared according to Tien *et al.*¹⁵⁸ by dissolving of 2.7g of Hydroxyethyl-cellulose (2000cP) in 96.3g of water containing 0.85g of sodium chloride and 0.1g of sorbic acid. The final pH was adjusted to 4.4 by adding sodium hydroxide, and the gel was stored at 2-8°C. General characteristics of the studied products are listed in **Table II. 1**. All products, except placebo and Replens®, are classified as medicines, whilst comprising an active pharmaceutical ingredient (API).

III.2.1.2. Materials

Reagents used include Roswell Park Memorial Institute-1640 RPMI (RPMI-1640, Sigma, Germany), Dulbecco's Modified Eagle Medium F12 (DMEM F12, Gibco, USA), Keratinocyte-serum free medium (Gibco, USA), human recombinant epithelial growth factor (hrEGF, Gibco, USA), bovine pituitary extract (BPE, Gibco, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Amresco, USA), Foetal Bovine Serum (FBS, Merck, Germany), Penicillin and Streptomycin (SP, Sigma, Germany), Phosphate Buffer Solution (PBS, Sigma, Germany), Dimethyl Sulfoxide (DMSO, Fisher Chemical, United Kingdom), Triton X100 (Fisher Chemical, United Kingdom), Sodium Dodecyl Sulphate (SDS, Acros Organics, Belgium), Nonoxynol-9/Tergitol (N9, Sigma, Germany), Neutral Red (NR, Acros Organics, Belgium), Ethanol (Manuel Vieira e C^a, Portugal), Glacial Acetic Acid (ChemLab, Belgium), Hydroxyethylcellulose (HEC; Natrosol 250 HX, Ashland Inc., USA), Sorbic Acid (Sigma-Aldrich, Germany) and Sodium Chloride (Merck, Germany). All used chemicals and reagents were of analytic grade or equivalent.

III.2.1.3. Epithelial cells

The cell lines HEC-1A, HeLa and VK2 E6/E7 were obtained from the American Type Culture Collection (ATCC-LGC Promochem, Teddington, United Kingdom). The uterine HEC-1A cells, originated from a line of human endometrial adenocarcinoma³²³, were cultured in RPMI 1640 supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% FBS, further referred to as RPMI complete medium (Passages 33-38). HeLa cell line is also epithelial, derived from

human cervical adenocarcinoma³²⁴. These cells were cultured in DMEM F12 medium supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), and 10% FBS and is further referred to as DMEM complete medium (Passages 64-68). VK2 E6/E7 cell line is also an epithelial one, derived from human vagina (HPV-16 E6/E7 transformed)³²⁵. VK2 E6/E7 cells were cultured in Keratinocyte-serum free medium (Keratinocyte-SFM) added of 0.1 ng/mL human recombinant epithelial growth factor (hrEGF), 0.05 mg/mL bovine pituitary extract (BPE) and calcium chloride 44.1 mg/L (Passages 4-6).

III.2.1.4. Vaginal tissues explants

Complete porcine genitalia, from approximately 6 month-year-old animals, were collected from a local slaughterhouse, transported under refrigeration to the lab and processed within 3h of animal death. The vaginal tubes were separated from the surrounding organs using scissors and tweezers. The vagina was cut longitudinally and the vestibule (caudal vagina) excised. The vestibule was selected since we found (unpublished data) to be the proximal vagina histologically more similar to the human one. Tissues were then washed on a prewarmed saline solution (NaCl 0.9%, 37°C). This section of tissue was then thickness normalized by using a manual dermatome (Watson Skin Graft Knife, BBraun, Germany) equipped with Aesculap blades (BA718Rm, BBraun, Germany). Then, epithelial sheets were placed upon aluminium foil (contact made by the basolateral side) and punched to sections of $\varnothing=3\text{mm}$ using a biopsy punch (Stiefel, GSK, United Kingdom). These sections were maintained on a warmed saline solution until use in the MTT assay. Tissues thickness was accessed to maintain homogeneity between samples. For this purpose, a digital micrometer (Vogel, Germany) was used by placing the tissue between two microscopy slides, measuring total thickness (µm) and then subtracting the thickness of the slides to the total measured thickness.

III.2.1.5. Testing products preparation

Testing products were diluted to 20, 5, 1, 0.4, 0.2, 0.1% (w/v), in medium without supplementation but containing 0.5% (v/v) of DMSO to ensure proper solubility of formulations. DMSO was selected based on the recommendations of the ISO guide¹⁹⁶. To assure that this substance was not itself cytotoxic, controls with the used concentration in culture media were performed. A negative control was included in all experiments (cells/tissues only with media/DMSO) and also positive controls for toxicity were included (SDS 5% (w/v), Triton X-100 1% (v/v) and N9 2% (v/v)). Formulations were left in contact with the testing platform (cell or tissue) for 24 hours.

III.2.1.6. Cellular toxicity

III.2.1.6.1. MTT assay

The MTT reduction assay was performed as previously described^{191,312,326,327} and ISO/EN 10993-5 guide for *in vitro* evaluation of vaginal medical devices¹⁹⁶. Cells were seeded onto 96-well plates (100.000 cells/mL) with the respective incomplete culture media. Cells were let to adhere for 24 hours at 37°C, 5% CO₂. After obtaining a half-confluent culture, 100 µL of testing formulations were added (see Testing products preparation section). After this period cells were washed with PBS and incubated for 4 hours with a 0.5 mg/mL solution of MTT reagent prepared in the respective culture medium without supplementation. Subsequently to formazan crystals formation, extraction was accomplished with 200 µL of DMSO for 15 minutes on an orbital shaker protected from light. Absorbances were then measured at 590 nm and 630 nm for background deduction, using a microplate spectrophotometer (BIORAD XMark, USA). Additionally, cells were photographed before and after the application of the MTT reagent solution in order to evidence the formation of the formazan crystals, using an inverted microscope (Olympus IX51, Japan, equipped with OCTAX Eyeware v.1.5 Build 406, Germany) - data not shown. Triton X-100, SDS and Nonoxynol-9 were used as positive controls, since they are widely known inducers of cytotoxic effects¹⁵⁹. The negative control consists of cells without any treatment (just culture media along the assay), which was considered as the 100% viability reference for products toxicity calculation. Further, the concentration that was toxic to half of the culture (cell/tissue), the half-maximal toxic concentration (TC₅₀), was calculated by logistic regression using GraphPad Prism Version 6.0 (Copyright, 2015).

III.2.1.6.2. NRU assay

The NRU assay was performed according to the literature^{191,195,328,329} and ISO/EN 10993-5 guide for *in vitro* evaluation of medical devices¹⁹⁶. Cells were seeded into 96-well plates (100.000 cells/mL) and maintained in culture until a semi-confluent monolayer was achieved (37°C, 5% CO₂, 24 hours). They were then exposed to the test compounds (100 µL) over the range of concentrations described above. After 24 hours of exposure, wells were washed once with 150 µL of prewarmed PBS, then 100 µL of NR medium was added to each well and the plates incubated at 37°C in a humidified atmosphere of 5% CO₂ for 3 hours. After incubation, the NR medium was removed, and cells were washed with 150 µL of PBS. Finally, 150 µL NR desorb solution (ETOH/acetic acid) were added to all wells, including blanks, in order to extract the dye. Plates were rapidly shaken on a microtiter plate shaker for 10 min to allow NR to be extracted from the cells forming a homogeneous solution. The absorbance of the resulting coloured solution at 540 nm was measured in a microtiter plate reader (BIORAD XMark, USA). NRU was determined for each concentration and compared to the one determined in control cultures. For the study a NR stock solution was previously prepared by dissolving 0.4 g of the dye in 100 mL of milliQ water. The NR medium was prepared in the day prior of usage by adding

1 mL of NR stock solution to 79 mL of culture media, followed by incubation overnight at 37°C and filtration through a 0.2 µm filter before adding to the cells, in order to be free of crystals. The NR desorb/extraction solution is composed of 1% glacial acetic acid solution, 50% ethanol and 49% water.

III.2.1.7. Tissue toxicity

III.2.1.7.1. Method optimization

Tissue explants were firstly tested for homogeneity and response to MTT assay. Therefore, tissues were submitted to a preliminary experiment, using different animals and applying the viability test on fresh and frozen tissues. Frozen explants were obtained as described (section “Vaginal tissue explants”) but tissue preparation occur after defrosting; upon reception from the slaughterhouse the vaginas were opened longitudinally (from vulva to cervix), washed in a HBSS solution pH 4.2 and frozen at -20°C, wrapped in aluminium foil, and stored in airtight bags. For viability studies (fresh tissue: 46 animals, at least n=2 for each animal, 6 independent experiments; frozen tissue: 12 animals, n=3, one experiment), tissues were left in culture for 24 hours and then the MTT assay was performed. For toxicity studies (only performed on fresh tissue: 23 animals, at least n=2 for each animal, 5 independent experiments) tissues were put in contact with SDS 5% (w/v) during 24 hours, and then the MTT assay has begun. SDS 5% is generally used as a positive control in tissue toxicity assays and recognized to have a toxic effect even on epithelial vaginal tissue³³⁰.

III.2.1.7.2. MTT assay

Tissue explants were placed in 96-well flat-bottomed tissue culture plates and treated with the testing formulations diluted on RPMI media without supplementation at 37°C, 5% CO₂ during 24 hours, n=6. Also, negative controls were included for each animal. SDS 5% (w/v), Triton X-100 1% (v/v) and N9 2% (v/v) were used as positive controls. Subsequently, tissues were washed twice with prewarmed PBS. MTT solution at a final concentration of 0.5 mg/mL was added to each well and incubated 1 hour at 37°C, 5% CO₂. Tissues were then transferred into new plates and extracted with 200 µL of isopropanol during 1.5 hours at room temperature under gentle shaking. Finally, tissues were discarded for plate reading at 590 nm against the background at 630 nm (BIORAD XMark, USA). The tissue viability was calculated as percentage from the negative control (tissue with no formulation).

III.2.1.7.3. Histological analysis

One tissue per formulation, at its highest concentration, 20% (w/v), was reserved for histological analysis. Tissues were fixed in a balanced 10% formalin solution. Subsequently, fixed tissues were run in a set of ethanol solutions of increasing concentrations for dehydration before embedding in paraffin. Tissue blocks were cut to have about 3 µm thickness and stained

with haematoxylin and eosin. Slides were observed and microphotographs were taken using a Zeiss microscope (Axiomager A1, Zeiss, Germany) equipped with a digital camera (Axiocam, Zeiss, Germany).

III.2.1.8. Data processing and statistical analysis

Data was analysed to produce arithmetic means with standard deviations (SD) using Microsoft Excel. Analysis of variance (ANOVA) and Dunnett's multiple comparisons test were performed to determine the significance of the difference between sets of data ($p < 0.05$). Logistic regression analysis was performed using GraphPad Prism 6.0 (Graph Pad Software, USA) to calculate the half-maximal toxic concentration (TC_{50}) with a 95% confidence interval in toxicity assays.

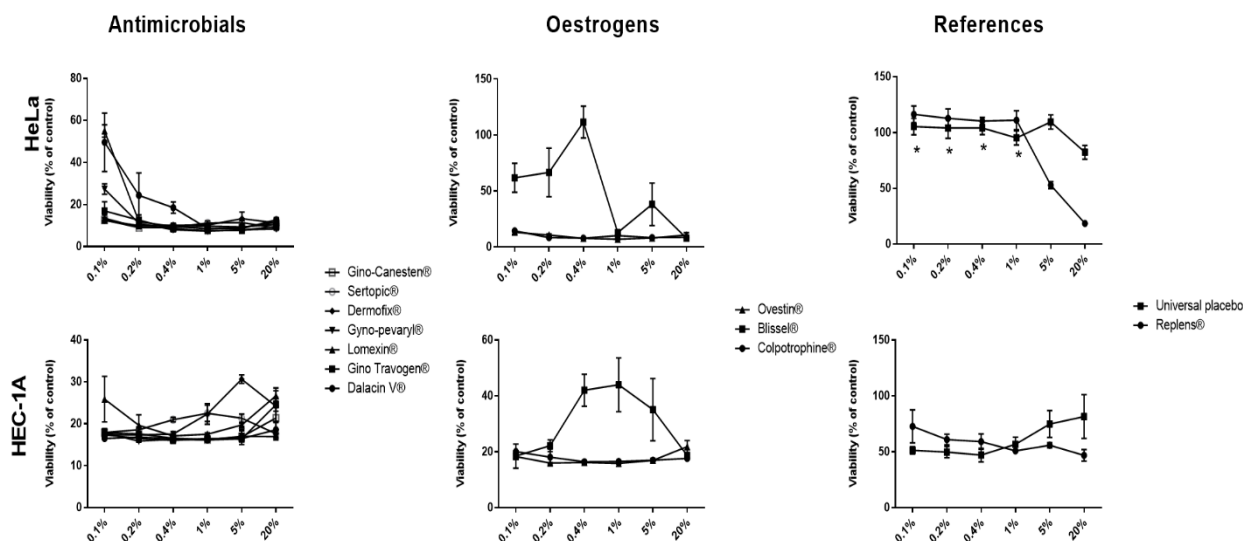
III.2.2. Results

III.2.2.1. Cellular toxicity

Cytotoxicity was accessed by means of the MTT reduction assay and NRU performed upon 3 different cell lines (HeLa, HEC-1A and VK2 E6/E7). Results are mean of 2 independent experiments, total n=6. All assays, were tracked microscopically for cell integrity, density and morphology, as stated in the respective protocol¹⁹⁶. Cell cultures acquired half-confluency and were able to reduce MTT in the mitochondria, and to integrate NR in the lysosomes. Results are expressed as viability percentage of the negative control (cells treated only with media). For all experiments, positive controls were used to assure the occurrence of induced toxic effects. In the NRU assay viabilities for HeLa and HEC-1A cells, of the positive controls (Triton X100 1%, SDS 5% and N9 2%) were: 2.75 ± 0.26 ; 3.62 ± 0.278 ; 3.79 ± 0.30 ; 17.23 ± 0.91 ; 29.75 ± 9.85 ; $19.10 \pm 1.82\%$, respectively. Standard deviations for negative controls (cells with media) were 10.93 and 16.30% for HeLa and HEC-1A, respectively. The results for NRU assay using the VK2 E6/E7 are not showed because they were inconclusive. In the MTT assay viabilities for HeLa, HEC-1A and VK2 E6/E7 cells, positive controls had viabilities of (Triton X100 1%, SDS 5% and N9 2%): 2.75 ± 0.26 ; 3.62 ± 0.28 ; 3.79 ± 0.30 ; 5.82 ± 0.34 ; 5.86 ± 0.322 ; $6.54 \pm 0.50\%$, $26.52 \pm 1.45\%$; 13.35 ± 3.93 ; and 22.12 ± 2.17 , respectively. Standard deviations for negative controls were 10.93, 11.51 and 14.63% for HeLa and HEC-1A and VK2 E6/E7, respectively. In **Figure III. 1**, all profiles for cytotoxicity are represented both for the NRU and the MTT assay. As a general picture, HEC-1A and VK2 E6/E7 cell lines conducted to higher viabilities than HeLa cells, being higher in the NRU assay. Antimicrobials showed a linear decrease of viabilities along with the increasing of products' concentration, as expected. Furthermore, this behaviour occurred consistently on the three cell lines and within the two assays, exception made for antimicrobials studied in HEC-1A using the NRU assay, in which viabilities do not seem to be affected by the dilution factor.

The oestrogen-containing formulations, Ovestin® and Colpotrophine®, presented the higher extents of cell toxicity, independently of the tested concentrations (just a slight increase was observed in the 0.1% concentration). Profiles for the same cell line were concordant between the assays. Blissel® presented an odd profile, having a high increase on viability at 5%, which is reduced at intermediate concentrations, and then at low concentrations conducts again to high viabilities. This behaviour might be related with the Blissel® polymeric composition (polycarbophil/carbomer) which, in the lower concentrations promote lower toxicity and then promotes somehow a negative effect on cell proliferation at intermediate concentrations (actually this dilution could promote a better diffusion of estriol). Concerning the reference products, low cytotoxic profile was observed, as expected. Universal Placebo gel led to most stable profile of viabilities across concentrations. Every concentration conducted to viabilities over 50% of cell viability.

A) NRU assay



B) MTT assay

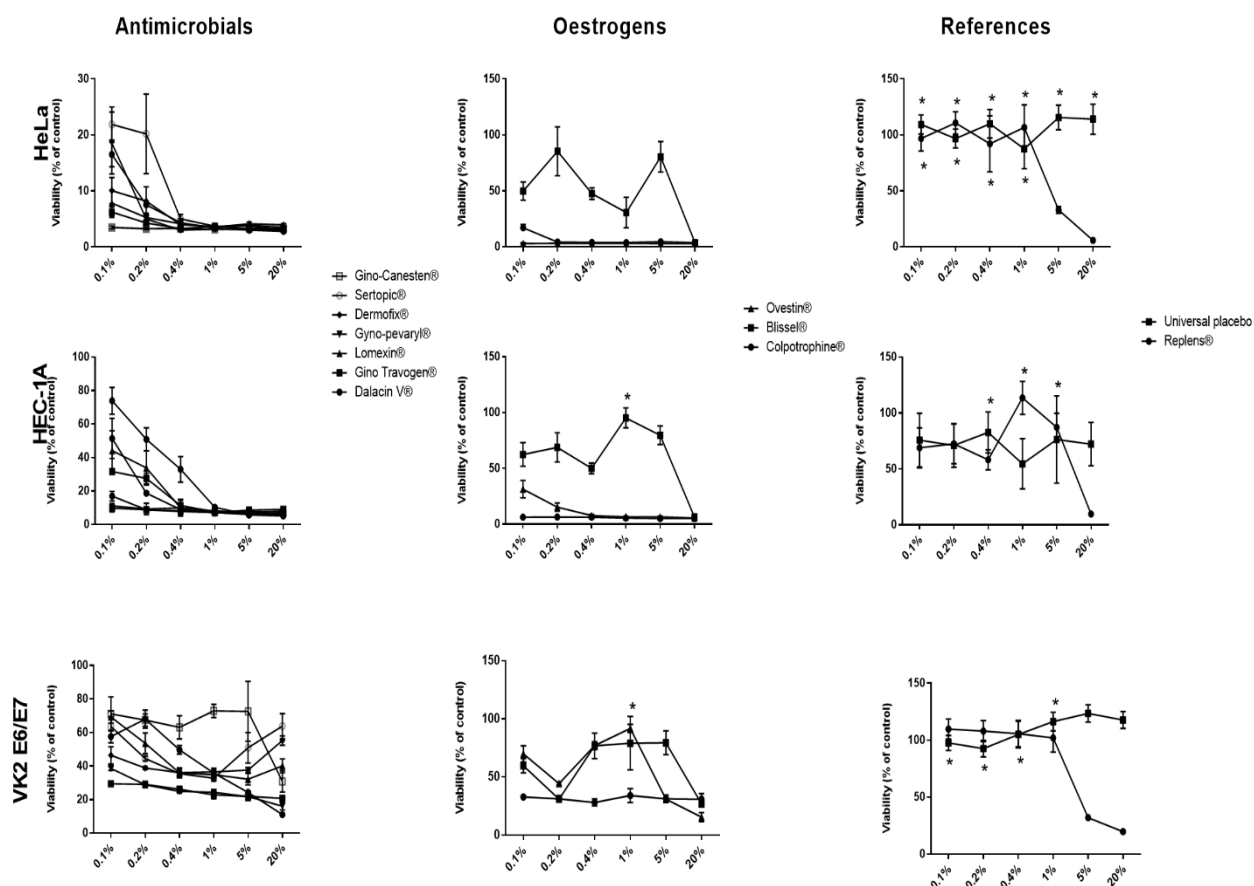


Figure III. 1: Cellular viability profiles for all the testing formulations at dilutions from 0.1 to 20% (w/v). Results for the NRU and the MTT assay. Viabilities are represented as percentage of the control treated only with culture media. Results are means and bars represent standard deviations from 2 independent experiments in which each condition was tested in triplicate (total n=6). * represents NO statistical difference from the control (two-way ANOVA, Dunnett's multiple comparisons test, $p < 0.05$).

On the other hand, Replens® only showed to reach high viabilities at concentrations lower than 5%. This fact may be related with low pH presented by this formulation, around 2.8, which together with a high pH-buffering capacity and high osmolality, could explain the high level of cytotoxicity presented³³¹.

Regarding VK2 E6/E7 cells, there are no studies available on the literature presenting results in the NRU assay, despite these cells being largely used to study vaginal toxicity mechanisms through other methods. In fact, we found irregular results with these cells on the NRU method (n=3, 2 independent experiments) and this could be due to an increased NR uptake induced by lysosomal swelling agents such as weakly basic substances and by osmotic swelling agents such as polyols, as was demonstrated in previous studies^{332,333}. Taking this into account, the lysosomal swelling may lead to an underestimation of the cytotoxicity when the NR assay is used^{195,314}.

III.2.2.2. Tissue toxicity

III.2.2.2.1 Method optimization

Optimization experiments were performed to assure that after collection and cutting procedures, tissues would have enough viability to be used in a toxicity assay such as the MTT reduction assay. Also, this step resulted in better handling of the surgical instruments and consequently more precise cutting technique for the operator. Results for viability and toxicity studies are shown on **Table III. 1**. Tissue viability was performed either on fresh and frozen tissue. As expected, frozen tissues returned low absorbances, which are not adequate to a final MTT assay, since this would have been the maximum absorbance to obtain, and could not distinguish significant differences between formulations. For this reason, performing such toxicity studies in frozen-thawed tissues is not possible. Furthermore, the performance of a toxic substance (SDS) confirmed that chemicals can induced negative responses in *ex vivo* tissues. *One-way ANOVA* with Dunnett's multiple comparisons test was applied to assess differences between the fresh tissue, frozen tissue and tissue treated with SDS, ($p < 0.05$ was accepted as denoting significance). On the fresh tissue experiment it was clear that high absorbances can be reached using an *ex vivo* model, i.e. suitable for toxicity testing of formulations. Moreover, an acceptable variation coefficient can be held with our tissue preparation technique and culture procedure (24.39%). After measuring tissues thickness (6 animals, n=6, 3 independent experiments) the variation coefficient was calculated to be 15.12%.

Table III. 1: Optimization and pre-validation studies on tissues viability and toxicity. The viability study corresponds to a negative control (tissues plus culture media) and the toxicity study was performed using SDS 5%, recognized to have a toxic effect on epithelial vaginal tissue. *Denotes significance on One-way ANOVA Dunnett's multiple comparisons test ($p < 0.05$).

Viability studies (Abs at 570nm) Culture media		Toxicity studies (Abs at 570nm) SDS 5%
Fresh tissue (mean±SD)	Frozen (mean±SD)	Fresh tissue (mean±SD)
0.5788±0.1412	0.1015±0.0144*	0.0581±0.0133*
Variation Coefficient: 24.39%	Variation Coefficient: 14.20%	Variation Coefficient: 22.83%
46 animals	12 animals	23 animals
n=2-3	n=2	n=2-3
6 independent experiments	1 experiment	5 independent experiments

III.2.2.2.2. MTT assay

Tissue viability results following exposure to the test products are shown in **figure III. 2**. Only Universal Placebo and Ovestin® were found to conduct to viabilities >50% by the first dilution. All other formulations, except Dalacin V®, returned viabilities above 50% upon the second dilution (1:20). Standard deviations were relatively high, but acceptable considering that the surrogate used for this test is biological. Positive controls, Triton X100 1%, SDS 5% and N9 2%, conducted to viabilities of 3.22±0.77, 2.51±0.81 and 2.56±0.60%, respectively, confirming that they have toxic effects on the vaginal epithelium (metabolic toxicity).

III.2.2.2.3. Histological analysis

Histological analysis performed on tissues after exposure to formulations was found to be a useful complement to tissue viability determination. Representative images of explants histology after exposure to the testing formulations are presented in **Figure III. 3**. All formulations seem to have induced epithelial alterations of the tissue when compared to the control. Nevertheless, oestrogenic (Ovestin®, Blissel®, Colpotrophine®) and reference products (Replens and Universal Placebo) were the ones to induce minimal changes. Also, Dalacin V®, an antibacterial, was able to preserve epithelial layers' integrity. On the other hand, antifungals showed extensive desquamation effects, being the most accentuated on Gino-Canesten®. As expected, positive controls (Triton, SDS and N9) conducted an extensive damage to epithelial layers. However, for Triton and N9 not as extensive as for SDS, which completely destroyed the epithelial and basal layers of the explant.

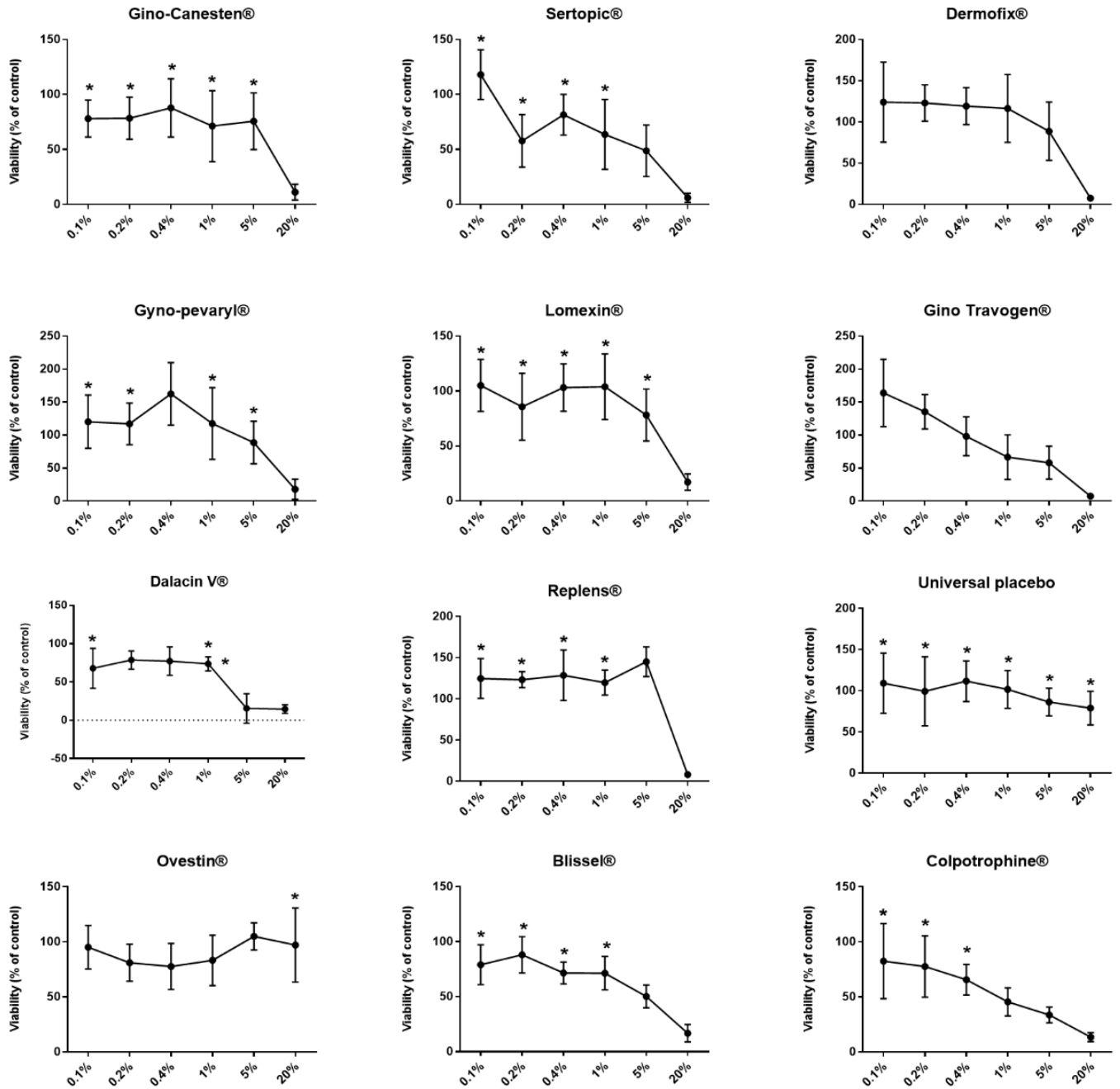


Figure III. 2: Tissue viability profiles for all the testing formulations at dilutions from 0.1 to 20% (w/v). Viabilities as percentage of the control tissue treated only with culture media. Results are means and bars represent standard deviations from 2 experiments in which each condition was tested in triplicate (total n=6). * represents NO statistical difference from the control (two-way ANOVA, Dunnett's multiple comparisons test, $p < 0.05$).

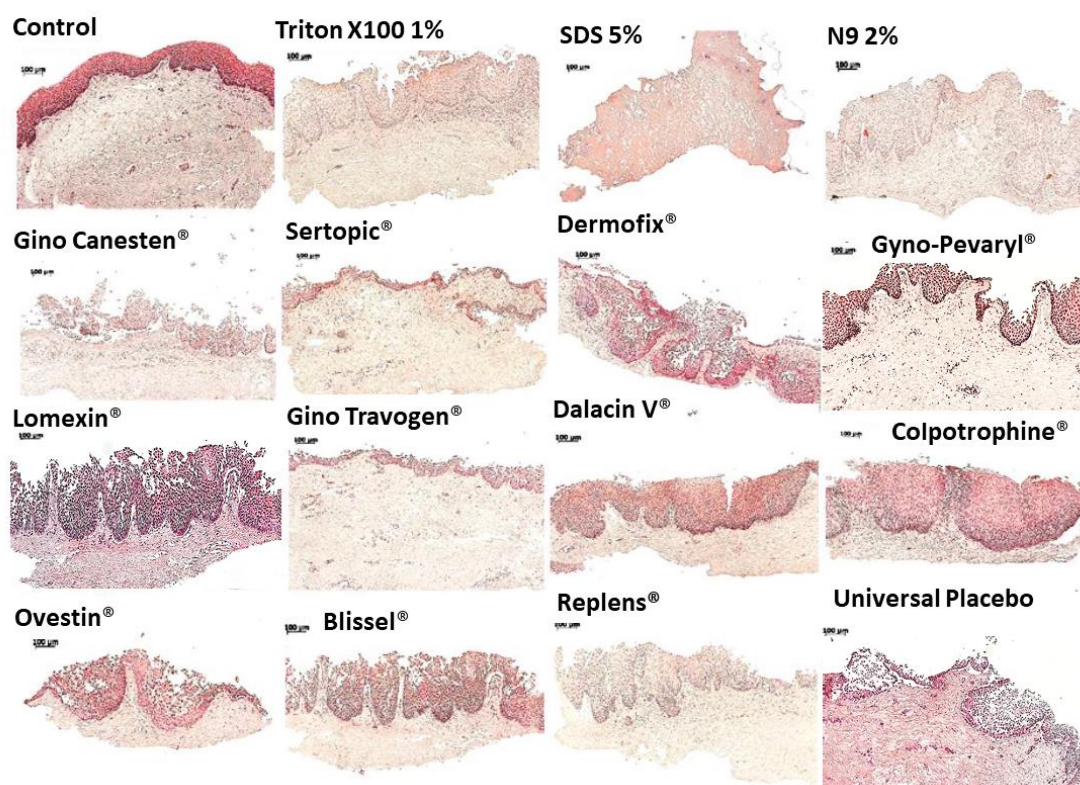


Figure III. 3: Impact of the tested formulations on the porcine *ex vivo* vaginal epithelium after 24 h of exposure. Histological images are representative of the higher concentration tested (20%), i.e. the worst-case scenario for the dilutions tested for tissue toxicity. H&E staining. Magnification 100x.

III.2.2.3. Models comparison

Calculated half-maximal toxic concentrations (TC_{50}) either for cellular models and tissue models are represented in **Table III.2** The *ex vivo* porcine vaginal tissue model, was the one that led to the calculation of the TC_{50} for almost all products. This means, on one hand that the formulations concentration in test is suitable to the test method, and on the other hand, that this method is the less sensitive in comparison to the remaining. Nevertheless, for Gino-Canesten® and Gino Travogen® the TC_{50} confidence interval was very wide, which could be related with the high slopes and SDs that are shown on the toxicity profiles for these products between concentrations 5 and 20%. The three cell lines were chosen because they were representative of three different epithelia that are present in the vaginal cavity, and, by testing these three cellular types we are addressing a complete toxicological profile of the formulation after administration by the vaginal route. Concerning the MTT assay, the most sensitive cell line was the HeLa (cervical cells), while VK2 E6/E7 (vaginal cells) was found to be the more robust. Since it is expected that cellular models are more sensitive to toxic effects than tissue models (due to the more complex and organized structure of the latter) and that the tissue, in turn, is more related to the *in vivo* environment, VK2 E6/E7 may provide more reliable results of the vaginal toxicity of formulations when performing the MTT assay. Neutral Red uptake assay should be performed to confirm previous results from the MTT assay, gathering the

maximum data for preclinical safety characterization of formulations, or to circumvent problems due to chemical incompatibility. When possible, final formulations, that were selected to proceed for further preclinical and clinical stages, should be tested under an *ex vivo* model, being the technique herein present a valuable alternative test method.

Table III. 2: Half-maximal Toxic Concentrations (% - TC₅₀) and confidence intervals (95%) calculated for cellular and tissue models using three different cell lines (HeLa, HEC-1A and VK2 E6/E7) and porcine vaginal explants. Within the cellular models, MTT and NRU assay were issued, as for tissues only MTT assay was performed.

Product	Cellular toxicity TC ₅₀ (%)			Tissue toxicity TC ₅₀ (%)		
	MTT assay			NRU assay		
	HeLa	HEC-1A	VK2 E6/E7	HeLa	HEC-1A	Porcine vaginal explants
Gino-Canesten®	<0.1	<0.1	7.039 (3.825-12.950)	<0.1	<0.1	18.530 (2.436-141.000)
Sertopic®	<0.1	<0.1	1.118 (0.550-2.272)	<0.1	<0.1	1.607 (0.568-4.549)
Dermofix®	<0.1	<0.1	5.243 (2.511-10.950)	<0.1	<0.1	>20
Gyno-Pevaryl®	<0.1	0.196 (0.114-0.336)	<0.1	<0.1	<0.1	>20
Lomexin®	<0.1	0.232 (0.142-0.381)	0.296 (0.198-0.441)	<0.1	<0.1	>20
Gino Travogen®	<0.1	0.141 (0.114-0.173)	<0.1	<0.1	<0.1	11.950 (0.949-150.400)
Dalacin V®	<0.1	0.974 (0.420-2.256)	0.386 (0.302-0.495)	<0.1	<0.1	1.233 (0.628-2.422)
Ovestin®	<0.1	<0.1	2.163 (1.206-3.882)	<0.1	<0.1	<0.1
Blissel®	0.579 (0.288-1.167)	6.249 (2.953-13.220)	8.008 (3.293-19.480)	0.825 (0.436-1.561)	<0.1	3.030 (2.000-4.591)
Colpotrophine®	<0.1	<0.1	<0.1	<0.1	<0.1	0.911 (0.591-1.405)
Universal placebo	>20	>20	>20	>20	2.982 (0.722-12.320)	>20
Replens®	5.186 (2.203-12.210)	10.000 (8.877-11.260)	4.847 (3.270-7.186)	5.731 (3.851-8.529)	>20	>20

III.2.3. Discussion

The vaginal epithelium is a common route for topical administration of antimicrobials, oestrogens, lubricants, and hygiene feminine-care products⁴. Nonetheless, these products could potentially conduct to acute irritation of the epithelial surfaces. Since the vaginal cavity can be used for either sporadic or chronic product applications, special attention should be taken when designing new products for this route. Viral and bacterial infection potentiation subsequent to the use of some vaginal lubricants has been demonstrated, reinforcing the need of extensive safety characterizations both for medicinal and cosmetic/hygiene products^{159,166,309,334-337}.

The standard preclinical test for the assessment of vaginal irritation and toxicity is the rabbit vaginal irritation (RVI) model. Similar to many other *in vivo* models, the reproducibility of results from this model (either between animals and between tests) is suboptimal. This variability is due to different responses of individual animals and to the trauma of insertion of the test product in the vaginal tract. Due to the high level of variability, standard RVI protocols use 10 rabbits per test article. The large number of animals required is undesirable from an animal welfare point of view and the variability decrease the confidence in the assay results. These problems are even magnified when primates are used for testing²¹¹.

Several *in vitro* and *ex vivo* models have been used to study the toxicity of substances and products, although it has not been stated which model better predicts *in vivo* toxicity³³⁸. On one hand, *ex vivo* assays are closer to fully represent the *in vivo* mechanisms. On the other hand, *in vitro* techniques are easier to handle, quicker, more sensitive and able for high-throughput screening in early steps of discovery and development. During product development, it has been observed that even slight modifications in final formulations, can either modify the efficacy and/or toxicity of a vaginal preparation^{186,339}.

Therefore, in our toxicity assays we assessed the toxicity profiles of final formulations belonging to different therapeutic classes (antimicrobials, oestrogens and reference products). The formulations included in this study are already commercialized and of course regarded as safe for human use, but our main purpose was to provide scientists involved in formulation with a rational tool that could rapidly disclose final products toxicity before getting to animal studies. Having that in mind, we were expecting that these finished products could, despite being safe, present some sort of *in vitro* toxicity. This actually means that poor results in *in vitro* stages of safety testing do not have to conduct to similar results *in vivo*. Some of the results herein present are somehow not clearly explained because the authors did not have access to the fully quantitative information of the formulations. This is the case of Blissel®, that presented an odd profile, although consistent in the two tests, models, and cell lines, a profile which is thought to be due to the presence of estriol. The behaviour presented is actually similar to the one that generally is seen for oestrogens *per se*. In fact, Blissel® is the oestrogenic formulation with the lower concentration of oestriol (50 µg/g vs 1 mg/g in Ovestin® and Colpotrophine®), and since the concentration in these last two is considered high, that might

explain the cytotoxic profile over all formulation dilution (even at 0.1%, the estriol content is able to saturate all oestrogenic receptors conducting to oxidative stress)³⁴⁰. Additionally, Blissel® showed an oestrogen-driven behaviour, conducting high cellular viabilities in low concentrations and low viabilities at high concentrations. Moreover, it was clear that the cellular models were affected by dilution effects (inconsistencies after certain dilutions), which do not represent a methodological issue in our *ex vivo* model. These findings along with the difficulties in attributing the toxic actions to a single substance, let us state that this screening of final formulation should be proceeded by individualized excipient and active substances responses. A study conducted by Gali et al (2010) exposed the toxic effects of various excipients for the vaginal route belonging to different classes, and the results obtained offer a useful guidance to select the most promising excipients displaying their toxicity *in vitro*. A criteria for selection appointed by the authors is that the concentration of a specific excipient should be below the CC₅₀¹⁸⁵. Transposing that assumption to this work we could estimate the product concentration on the vaginal fluid at each moment - 0.75 mL³⁷ and, adjust the recommended doses in order to minimize toxicity, although warranting the therapeutic effect. Likewise, Gali *et al.*, we found interesting correlations between the data obtained in the various assays. This implies that the different assays do not generate independent data, although the sensitivities can differ. Therefore, to begin with screening, the simplest assay should be preferred. Thereafter, the most promising formulations could then be confirmed in a more relevant model, such as the *ex vivo* model, and after that an *in vivo* model.

It is well established that data obtained using *in vitro* models during the preclinical stages of development will not match entirely the outcomes in clinical trials. This happens because, firstly, the settings are different on the two stages (API concentration and length of exposure and, of course, the biological environment), and, secondly, the parameters evaluated in clinical trials are wider. Such as, histopathological evaluation, assessment of the vaginal inflammatory condition, pH and microflora appreciation. Consequently, while there is no certainty about the *in vivo* standard for safety assessment, potential safety issues detected on *in vitro/ex vivo* assays should be seriously considered. Several international research groups and organizations have reunited efforts to identify vaginal biomarkers, including cytokines and chemokines, in an attempt to correlate *in vitro* and *in vivo* safety testing more properly. Fichorova *et al.* in 2001, have established a correlation between mucosal toxicity and increased levels of the proinflammatory chemokine IL-8 in vaginal washings of spermicide-treated rabbits³⁴¹. Thus, quantification of IL-8 might be used as a sensitive analyte to complement *in vitro* toxicity testing.

It is clear that before beginning product evaluation, all excipients and APIs should be checked for biocompatibility. The product evaluation comes next to reinforce the non-toxic effects after the formulation step. Testing semisolids formulations (i.e. final formulations) toxicity using both *in vitro* and *ex vivo* models has previously been performed. For example, Rohan *et al.* showed that a tenofovir gel and a placebo gel composed of hydroxyethylcellulose, EDTA, citric acid, glycerin, and the preservatives methyl- and propylparaben were detrimental

toward epithelial cells and explants, causing a reduced viability and epithelial layer integrity of HEC-1A and Caco-2 cells and cervical tissue explants³⁴². Similar observations were made by Dezzutti *et al.*, assessing the toxicity and epithelial layer integrity of HEC-1A and Caco-2 cell lines after exposure to PRO-2000 gel, UC-781 gel, and the placebo gels methylcellulose and Vena Gel³⁴³. Furthermore, our workgroup has already tested several vaginal lubricants commercially available, under a safety perspective, concerning cytotoxicity, pH and osmolality¹⁵⁹. Earlier a similar study has been performed by Dezzutti *et al.* on 10 aqueous-based lubricants to test not only cellular toxicity and damage to epithelial monolayers, but also their toxicity upon human explants epithelium. Main findings of toxicity to the microflora were attributed to preservatives contained in the formulations. Alongside, it was concluded that epithelial trauma alone would not be sufficient to increase the HIV-1 transmission, having inflammatory response an important role in this³³⁷. Similarly, to the study herein presented these authors refer that they had some difficulties in performing the toxicity tests in the cellular models, because, when testing creams (which are composed of an oily phase and an aqueous phase) it was difficult to solubilize and wash the cells during the tests. Actually, we have circumvented this problem by using 0.5% (v/v) of DMSO in the final formulation dilution with culture media to prevent phase separation of these type of products in test. Furthermore, with handling practice and constant microscopy monitoring, we were able to check for cell detachment after washing steps (which we concluded that do not happened). Still, it was also observed that washing steps were even easier when performing the tests on the *ex vivo* model.

In 2006, MatTek™ Corporation, an American enterprise specialized in developing and producing organotypic models, i.e. reconstructed tissue models for drug/product testing concerning several routes of administration, published an *in vitro* vaginal human tissue model with low variation coefficients intra and inter-batch (<10 and <15%, respectively), being an innovative and improved toxicological test system²¹¹. In comparison to *in vivo* models, this system (EpiVaginal™) reduces the testing duration, allows for high-throughput screening, and complies with the “3Rs” policy leading to the decrease of number of animals used to screen feminine-care products. Besides, it could be applied to vaginal drug delivery assays, bacterial adhesion and omics³⁴⁴. EpiVaginal™ is available as an epithelial tissue (grown from normal human vaginal epithelial cells) and as a full thickness tissue (including epithelial cells and a fibroblast-containing *lamina propria*). These two tissues could also include immune-competent dendritic cells for inflammatory studies. Another company providing researchers with this type of tools in Europe is EpiSkin™, producing HVE (reconstructed human vaginal epithelium) that is composed of A431 cells (derived from a vulvar epidermoid carcinoma). It is well established that these tools are clearly advantageous when in comparison with other *in vitro* and *ex vivo* models, not only in terms of sensibility and reproducibility, but also in terms of technology, quality control and even technical assistance. Nevertheless, they have main applicability to industry screening platforms, rather than academic research, because of the economical expenditure. That is a key point to understand why *ex vivo* tissues could represent valuable tools for scientific research, when early stages of the preclinical development of

drugs/products is the focus. Moreover, the *ex vivo* models take into consideration an interindividual variability that is not present in manufactured reconstructed models, and that in fact, is closer to the variability encountered further on *in vivo* studies.

Despite having a full cell structure (epithelial, connective, immune) and better tolerance to formulations, tissue explants also have some drawbacks like variability, and the fact that they are technically more demanding. Also, *ex vivo* tissue is not entirely representative of the *in vivo* situation due to lack of tissue regeneration, lack of immune cells recruitment, and independence from hormones^{337,342}. If human explants are used, they can also be of limited number and require an Institutional review board approval²¹⁷.

In our study, a good overlapping profile could be found between the cellular and the tissue model, although cellular testing was hypersensitive, which conducted to lower viabilities, turning impossible the calculation of TC₅₀. Indeed, the ability to calculate TC₅₀ for almost all formulation in the *ex vivo* model indicates that this model is valuable to assure direct comparisons, even allowing the use of more concentrated dilutions of formulations, if experimentally possible.

The *ex vivo* porcine tissue model herein presented extends the use of *ex vivo* porcine tissue as a surrogate for human vaginal tissue. There is a great deal of data that validate the porcine model of vaginal mucosa in terms of structure, function and reactivity in comparison to human tissue. Both have stratified squamous epithelium supported by connective tissue. The use of small, *ex vivo*, specimens provides convenience, efficiency, and high throughput for screening^{34,39}. Samples of porcine tissue are inexpensive to obtain (in abundance on slaughterhouses) and handling is easy when compared to the use of whole animals^{34,42}. *Ex vivo* porcine tissues have been largely tested for drug permeation. But, despite appearing to be a good *ex vivo* permeability model for human vaginal tissue extrapolation it has already been shown that its reliability may vary upon substances chemical characteristics. For example, for hydrophilic molecules, (water and vasopressin, for example), the porcine vaginal tissue is an accurate *in vitro* permeability model of human tissue, while for more lipophilic molecules (such as oxytocin) the flux could be higher than the corresponding estimated value for human tissue²⁴⁶. Another limitation related to the *ex vivo* vaginal model is the fact that some authors use cervical and uterine explants, to resemble the vaginal epithelia^{185,197,342,345}. This may be due to the difficulty in accessing human vaginal epithelium and/or also based on the assumption that vaginal formulations shall be safe not only to the vagina but also to the uterus. The porcine model can relatively circumvent this problem of accessing specimens for testing and provide a better similitude with the human vagina itself. Added to that, tissue collection and culture procedures had not been standardized and optimized, before this research. Previous studies of ours have been focused on disclosing the likeliness of porcine vaginal tissue and the human tissue, and even disclosing which anatomical region of the porcine vagina is more appropriate to collect in order to comply with human similarity.

The tetrazolium-based MTT assay has long been regarded as the gold standard of cytotoxicity assays as it is highly sensitive and has been miniaturised for use as a high-

throughput screening assay. The first use of the MTT assay upon tissue explants goes back to the 90's, when it was applied to different types of tissues, for instance buccal mucosa^{346,347}. This method is also the one recommended by the manufacturers in the EpiVaginal™ and HVE™ reconstructed models. Recently, Van Tonder *et al.* (2015), conducted a study comparing several toxicity assays, including the MTT and NRU assays in MDA-MB-231, MCF-7 and MCF-12A cell lines. Results indicated that the NRU showed one of the smallest variability across the linear range, while the largest variation was observed for the MTT assay. This implies that this assay would more accurately detect small changes in cell number than the MTT assay. Furthermore, the MTT assay was the one to have more interferences with test products³⁴⁸. Although seeming less sensitive and with higher number of interferents, MTT has a large historical use, and researchers are familiar with the methodology. Furthermore, standard documents like those from the “International Organization for Standardization”, specifically the 10993-5 normative, recommend this procedure for cytotoxic assessment of medical devices¹⁹⁶. Moreover, it has a successful history of application in tissues.

III.2.4. Conclusions

In vitro and *ex vivo* models have been applied to assess the safety profiles for drugs, excipients and final formulations in order to early preview toxicity issues that might arise in the ensuing steps of product development. Herein we presented some preclinical toxicity results obtained for a panel of vaginal semisolid formulations, already regarded as safe for human use. Furthermore, we have not only optimized a strategy to perform *in vitro* cellular toxicity assays using semisolid aqueous and non-aqueous formulations; but also, developed a collecting technique for vaginal *ex vivo* porcine tissues. The later showed to be a possible strategy for *ex vivo* toxicity testing, since it demonstrated acceptable reproducibility and sensitivity, with relatively low variability. The inclusion of this method in preclinical safety assessments may optimize cost-efficiency of new formulations development by predicting efficacy and safety profiles at early stages of product development. It could not only be applied to the pharmaceutical industry and research, but also to the cosmetic, hygiene, and medical devices industries. Moreover, it could further be applied to the development of primary cell cultures, as a surrogate for permeation and metabolic studies; and to originate co-cultures with microorganisms.

III.3. EXPERIMENTAL

III.3.1. Materials and Methods

III.3.1.1. Chemicals and testing products

For the preparation of the assay controls, the following chemicals were used: sodium chloride (NaCl, JT Baker, USA), sodium dodecyl sulphate (SDS, Acros Organics, Belgium), nonoxynol-9/tergitol (N-9, Sigma, Germany), sodium hydroxide (NaOH, VWR Prolabo, Germany) and type I water (Millipore, Merck, USA).

The testing products included in this study were vaginal semisolids, available in the international market, intended both as therapeutics for several pathological conditions, and as lubricants for sexual and menopausal discomfort. The ten different therapeutic products were those tested in the previous chapters **Table II. 1**. The lubricants tested were: Fillergyn® gel (BSDpharma), Geliofil® Classic gel (Laboratoires Effik), GelSea® gel (LDPSA), Ginix® gel (ISUS), Ginix® Plus gel (ISUS), Hyalo Gyn® gel (Fidia Farmaceutici), K-Y® Jelly (Johnson & Johnson), Phyto Soya® gel (Arkopharma Laboratoires Pharmaceutiques), Velastisa® Intim VG moisturizer gel cream (ISDIN) and Vidermina® gel (Istituto Ganassini). Both Replens® (Laboratoires Majorelle) and Universal Placebo¹⁵⁸ were used as controls, since their toxicity profiles are largely described on the literature^{14,93,153,158,206,233,272,321,322}. Universal Placebo was prepared as described on experimental section of Chapter II. To evaluate the method sensitivity to vaginal irritants, nonoxynol-9 was used in concentrations ranging from 0.001 to 100% (v/v).

III.3.1.1.1 Eggs and incubation conditions

The test system consists on Leghorn white hen's white eggs, fresh (not more than 7 days old), clean, weighing 45 to 65 g. When the eggs arrived to the lab, they were checked for damages in the shell: damaged eggs were discharged while the others were incubated, at $37.8 \pm 0.3^\circ\text{C}$ in an atmosphere with a relative humidity of $58 \pm 2\%$ and under automatic rotation, during 8 days (Corti AF-50 and Copele 30652, Spain). By the eighth day, eggs were inspected using a LED light to confirm the embryo formation. Non-embryonated eggs were discarded and the others were incubated for 1 one more day in the same conditions but without egg rotation.

III.3.1.1.2. HET-CAM assay

The assay was conducted according to the ICCVAM - Recommended test method (NIH Publication No. 10-7553 - 2010). At day 9, the eggs were taken out of incubator and were placed on an appropriate support with the larger part facing up. The shell was opened with the help of a scalpel and tweezers and the chorioallantonic membrane (CAM) was exposed. This membrane was then hydrated with NaCl 0.9% (w/v) for a maximum of 30 min. Afterwards, the solution was aspirated and the membrane was peeled off without damaging the blood vessels. For each product 0.3 mL were applied on the membrane and 3 eggs per products were used. The irritant effect of these products was evaluated by monitoring the appearance of three endpoints in the CAM, for 5 minutes: haemorrhage (vessel bleeding), lysis (vessels

disintegration) and coagulation (protein denaturation intra and extra- vascular). In the present study, these endpoints were evaluated accordingly to two different criteria: Irritation Score (IS) A and B. While for criteria A the endpoints were checked at predetermined time intervals (0.5; 2 and 5 min), for criteria B these effects were monitored continuously during 5 minutes paying attention to the time when the irritant response begun which was registered. This methodological difference conducts to different range of categories in the irritant outcome (Table III. 3). Photographs were taken at the beginning and end of the assays. Calculation of the IS for each test product is represented as mean \pm standard deviation (SD) of a total of three eggs.

Table III. 3: Irritancy classification. Classification on the *in vitro* HET-CAM assay concerning Irritation Score (IS) analysis methods A and B.

Analysis method A					Analysis method B	
Irritation response	0 to 8: Non-severe irritant 9 to 21: Severe Irritant				0 to 0.9: Non-irritant 1 to 4.9: Slight Irritant 5 to 8.9: Moderate Irritant 9 to 21: Severe Irritant	
IS calculation method	Calculation of the IS is the sum of the scores attributed at each time point to the arising of the corresponding effect, as stated on the following scheme:				Calculation of the IS by applying the following equation:	
	Endpoint	Score			$\left[\left(\frac{(301 - Hemorrhage\ time)}{300} \right) \times 5 \right]$	
		0.5 min	2 min	5 min	$+ \left[\left(\frac{(301 - Lysis\ time)}{300} \right) \times 7 \right]$	
	Lysis	5	3	1	$+ \left[\left(\frac{(301 - Coagulation\ time)}{300} \right) \times 9 \right]$	
	Hemorrhage	7	5	3		
	Coagulation	9	7	5	Times should be replaced by the time (in seconds) at which each effect started.	

III.3.2. Results

The irritation potential determined for N-9 using the HET-CAM assay was reasonably comparable when using both evaluation tools, IS (A) and (B), as it can be observed in **Figure III. 4**. Only at the concentration of 0.5% (w/v) the difference between these two testing criteria results was statistically different (two way-ANOVA, $p < 0.05$, Sidak's multiple comparisons test), although standard deviations were relatively high. This concentration seems to be the one that presents a *borderline*-type behaviour, since its score (on criteria B) is in the limit between slight and moderate irritant. The same might be happening for concentration 1% which is in between moderate and severe irritant, on the IS(B) scale. It can be also observed that concentrations above 2% were the ones that gave IS values higher than 9, meaning that at these concentrations N-9 exhibits severe vaginal irritation effect (IS (A) $N-9\ 2\% = 10.0 \pm 0$; IS (B) $N-9\ 2\% = 10.8 \pm 0$). Taking into consideration the IS (B) criteria, concentrations between 0.3 and 1% were classified as moderately irritants (IS (B) $N-9\ 0.3\% = 4.8 \pm 0.5$; IS (B) $N-9\ 1\% = 8.7 \pm 0.4$). Concentrations between 0.01 and 0.2% were slight irritants (IS (B) $N-9\ 0.01\% = 3.7 \pm 0.6$; IS (B) $N-9\ 0.2\% = 4.9 \pm 0.7$). The two lowest concentrations tested, 0.001 and 0.005%, were regarded as non-irritants, having scores of 0.9 ± 0.8 and 1.1 ± 1.0 , respectively. In what concerns to the IS (A) criteria, the non-severe irritant response was found for concentrations ranging from 0.001 and 1 with scores of 0.7 ± 0.6 and 7.3 ± 1.2 , respectively. These results show that the most irritant N-9 concentration is found independently of the scale being used. Nonetheless, the IS (B) is able to discriminate more irritation categories when compared to IS (A).

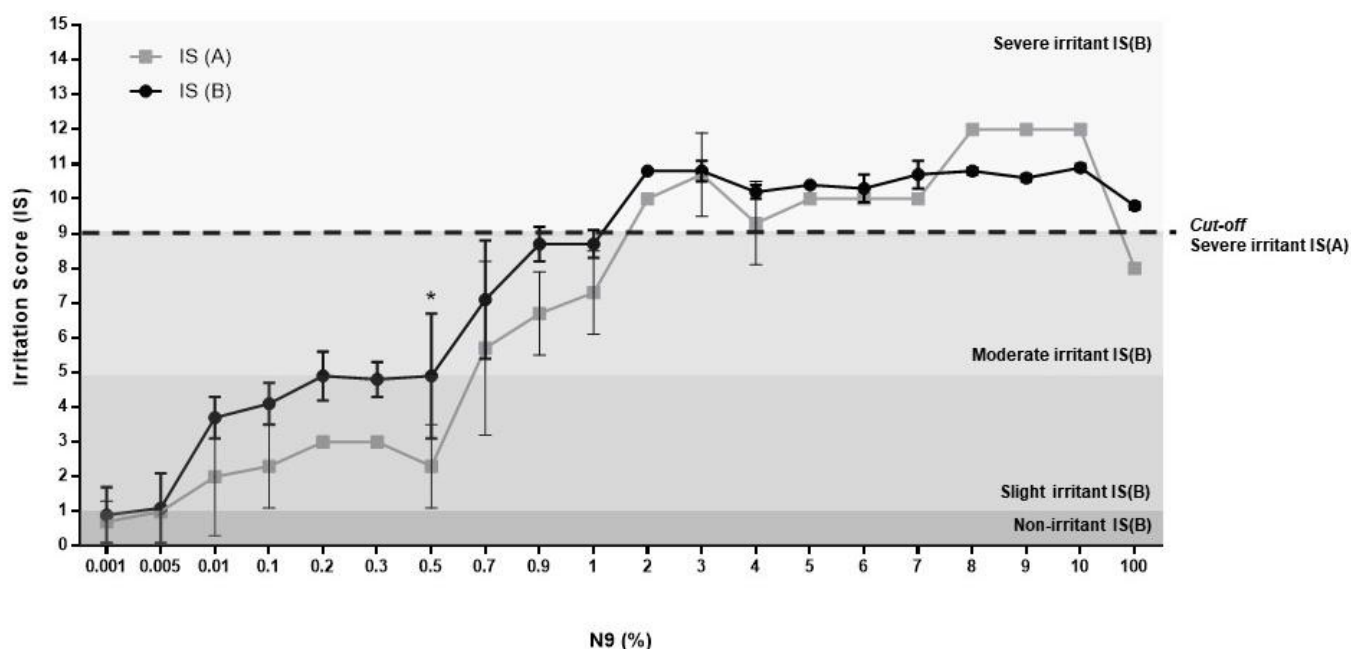


Figure III. 4: Irritation Scores (IS) for N-9 (nonoxynol-9) according to categories A and B. Results are presented as mean values and bars as standard deviation (n=3). * denotes statistical difference between the two scales IS (A) and IS (B) (two way-ANOVA, $p < 0.05$, Sidak's multiple comparisons test).

The irritation scores determined for the vaginal formulations are represented on **Figure III. 5**. Concerning the therapeutic products **Figure III. 5 (a)**, Universal Placebo, Replens®, Dermofix®, Sertopic®, Dalacin V®, Ovestin® and Blissel® did not conduct to any irritant response, having been scored with 0 ± 0 . Gyno-Pevaryl®, Gino-Canesten® and Colpotrophine® had significantly different scores when evaluated with the two criteria. Concerning criteria A, they were all classified as non-severe irritants. However, when the criteria B was used, Gino-Canesten®, Colpotrophine®, Gyno-Pevaryl® were classified as slight irritants ($IS (B)_{Gino-Canesten} = 3.3 \pm 0.3$; $IS (B)_{Colpotrophine} = 2.0 \pm 0.0$; $IS (B)_{Gyno-Pevaryl} = 4.8 \pm 2.0$). All except Gino Travogen®, had scores higher in scale B than on scale A ($IS (A)_{Gino Travogen} = 3.0 \pm 0.0$; $IS (B)_{Gino Travogen} = 2.0 \pm 0.6$), although this difference was not statistically supported.

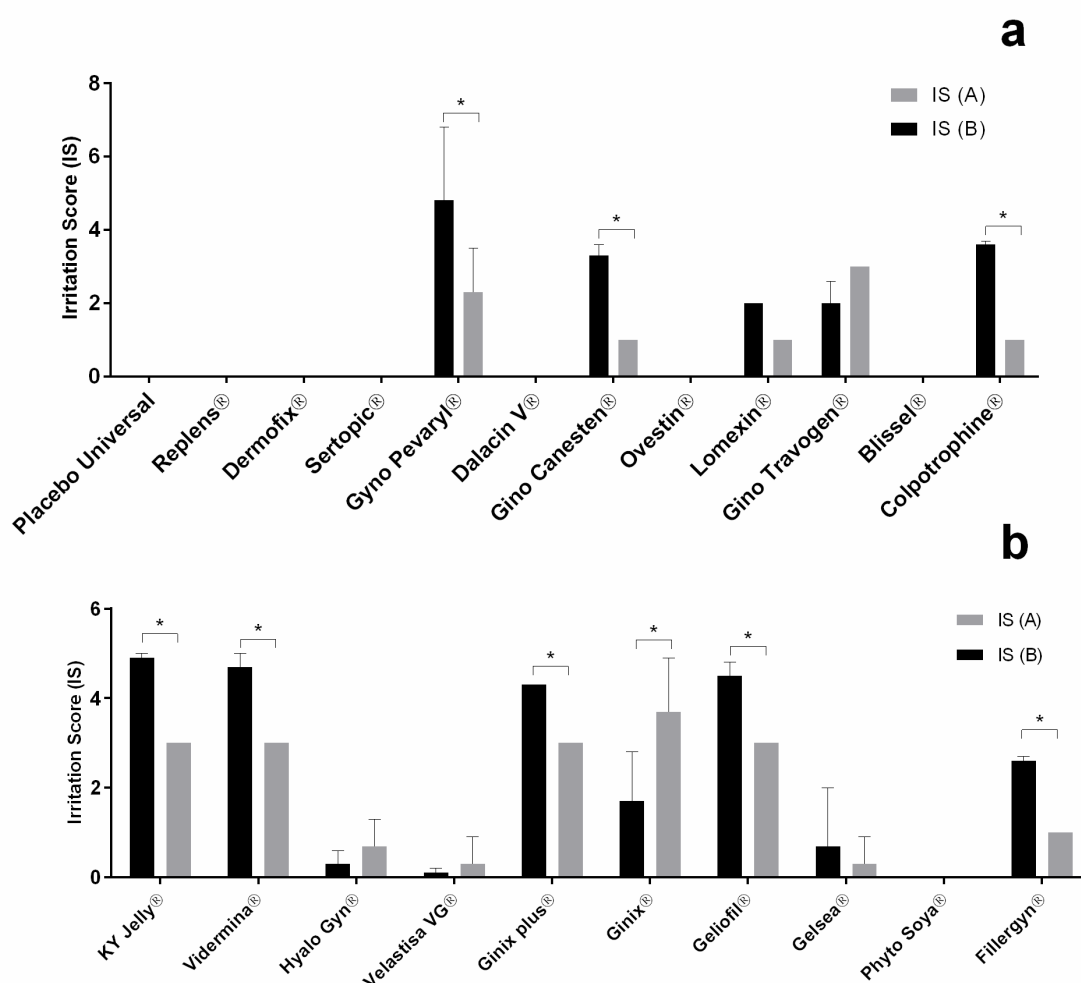


Figure III. 5: Irritation Scores (IS) for therapeutic vaginal products (a) and vaginal lubricants (b) according to the categories A and B. Results are presented as mean values and bars as standard deviation ($n=3$). * denotes statistical difference between the two scales IS (A) and IS (B) (two way-ANOVA, $p < 0.05$, Sidak's multiple comparisons test).

Regarding the vaginal lubricants (**Figure III. 5 (b)**), only Phyto Soya® had a score of zero. Hyalo Gyn®, Velastisa VG® and Gelsea®, which showed no significant difference when comparing both scales, also obtained the least irritant scores. The remaining products had significant differences between the evaluation of IS (A) and (B). The remaining products had

significant differences between the evaluation of IS (A) and (B). All lubricants were classified as non-severe irritants by the IS (A) irritation criteria with Ginix® reaching the highest score in this scale (3.7 ± 1.2). When the IS (B) was applied Hyalo Gyn® and Velastisa VG® were classified as non-irritants and all the others were classified as slight irritants. For all but Ginix®, the score obtained in scale B was higher than the one obtained in A. As it can be seen on **Figure III. 5 (b)**, when comparing both scales, they also obtained the least irritant scores. The remaining products had significant differences between the evaluation of IS (A) and (B). **Table III. 4** details the score obtained and the classification attributed to all products tested in this study. Furthermore, a picture from the test end-time (5 min) is also shown for products and controls. As it can be observed, some products did not present the same classification when comparing the two scales (*borderline* behaviour). In the case of medicines, Gyno-Pevaryl®, Gino-Canesten®, Lomexin®, Gino Travogen® and Colpotrophine®, were the ones to present a classification of slight irritants on IS (B) and non-severe irritants on IS (A). Regarding lubricants, K-Y Jelly®, Vidermina®, Ginix Plus®, Geliofil® and Fillergyn®, presented this behaviour.

Table III. 4: Irritation potential. Irritation scores, IS (A) and (B) determined for vaginal semisolid medicines and lubricants.

Medicines				Lubricants			
Product		IS (A)	IS (B)	Product		IS (A)	IS (B)
Placebo Universal		Non-severe irritant (0)	Non-irritant (0)	K-Y Jelly®		Non-severe irritant (3)	Slight irritant (4.9)
Replens®		Non-severe irritant (0)	Non-irritant (0)	Vidermina®		Non-severe irritant (3)	Slight irritant (4.7)
Dermofix®		Non-severe irritant (0)	Non-irritant (0)	Hyalo Gyn®		Non-severe irritant (0.7)	Non-irritant (0.3)
Sertopic®		Non-severe irritant (0)	Non-irritant (0)	Velastisa VG®		Non-severe irritant (0.3)	Non-irritant (0.1)
Gyno-Pevaryl®		Non-severe irritant (2.3)	Slight irritant (4.8)	Ginix Plus®		Non-severe irritant (3)	Slight irritant (4.3)
Dalacin V®		Non-severe irritant (0)	Non-irritant (0)	Ginix®		Non-severe irritant (3.7)	Slight irritant (1.7)
Gino-Canesten®		Non-severe irritant (1)	Slight irritant (3.3)	Geliofil®		Non-severe irritant (3)	Slight irritant (4.5)
Ovestin®		Non-severe irritant (0)	Non-irritant (0)	Gelsea®		Non-severe irritant (0.3)	Slight irritant (0.7)
Lomexin®		Non-severe irritant (1)	Slight irritant (2)	Phyto Soya®		Non-severe irritant (0)	Slight irritant (0)
Gino Travogen®		Non-severe irritant (2)	Slight irritant (3)	Fillergyn®		Non-severe irritant (1)	Slight irritant (2.6)
Blissel®		Non-severe irritant (0)	Non-irritant (0)				
Colpotrophine®		Non-severe irritant (1)	Slight irritant (3.6)				
Assay controls							
Product		IS (A)	IS (B)	Product		IS (A)	IS (B)
N-9 2% (v/v) (Positive control)		Severe irritant (10)	Severe irritant (10.8)	NaOH 0.1N (Positive control)		Severe irritant (19)	Severe irritant (19)
SDS 1% (w/v) (Positive control)		Severe irritant (10)	Severe irritant (10)	NaCl 0.9% (w/v) (Negative control)		Non-severe irritant (0)	Non-irritant (0)

III.3.3. Discussion

Nowadays, the increasing number of new chemicals and products introduced into the market coming from several industries (cosmetic, pharmaceutical and medical devices) has generated the need to validate *in vitro* techniques able to screen potential irritation effects at the early stages of development.

The Hen's Egg Test, or Hühner-Embryonen-Test (HET), firstly presented by Luepke, back in the 80's, was developed to be a rapid, sensitive and inexpensive toxicity test able to provide information on embryotoxicity, teratogenicity, systemic, metabolic and immunopathological effects³⁴⁹. This method was designed to be applied in mucous-membrane irritation testing, and the tool provided for score and classification was analogous to the Draize test. Since then, it has been confirmed that a good correlation between these two tests, i.e. *in vitro/in vivo* correlation, for plenty of chemicals do exist. The HET-CAM assay cannot totally replace the irritation tests in mammals, but can largely decrease the number of animals used, and limit the pain and injury inflicted to animals during experiments³⁴⁹.

The application of the HET-CAM assay to vaginal irritation testing comes in line with the referred compliance with the 3R's policy. In the vaginal products field, there is no organotypic irritation test approved neither on the EU, nor the USA. In fact, there are some cellular and tissue models available for toxicity testing, however mainly comprise techniques for specific metabolic pathways, histological analysis and inflammatory response^{42,195,330,350}. Our research group is focused in developing strategies for preclinical safety characterization of vaginal products using cellular and *ex vivo* tissues assays (paper submitted for publication). The usage of HET-CAM assay in vaginal products testing comes to widen the safety assessment portfolio that can be applied to test substances or products in a quicker and more effective way on the first step of preclinical safety testing. In this study, several semisolid vaginal medicines and lubricant products were tested. Moreover, two analytic methods of the IS were applied. It was concluded that when testing N-9, a pure substance, there was no statistical difference when applying one analytic method or another. Actually, the concentration found to be the one that could trigger severe irritant effects, 2% (w/v), was the same that was shown to generate severe toxicity in clinical trials, being less safe than thought at preclinical evaluations³⁰⁹. In that case, N-9 was being studied as a spermicide, but, because of its surfactant nature, it ended up on being irritant, and even promoting the transmission of HIV infection³⁵¹⁻³⁵³. Having this episode in mind, the need for more appropriate *in vitro* assays to predict *in vivo* safety issues, is even more highlighted. In our study, the surfactant nature of N-9 may be responsible for the fact that when increasing abruptly its concentration (up to the pure substance) no higher rate of irritation was observed (U.S. Public Health Service, Department of Health and Human Services, 2006). Universal placebo, on the other hand, was herein included because it is a vaginal gel that was wittingly design to be a control formulation in clinical trials of vaginal microbicides. Its safety profile, already confirmed by clinical trials, was once more confirmed in this study, having an IS of zero on the two scoring grids (A and B).

Concerning semisolid vaginal medicines, no severe irritant responses were observed, and this outcome was already expected. They are already commercialized and since they are classified by the competent authorities as medicines, they were not only subjected to preclinical evaluations, but also to extensive clinical trials. Moreover, the products herein classified as non-severe irritants (IS (A)) and slight to moderate irritants (IS (B)), such as Gyno-Pevaryl®, Gino-Canesten®, Colpotrophine®, Gino Travogen® and Lomexin® were also tested by our research group using two *in vitro* cytotoxicity tests: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and neutral red uptake (NRU) into uterine (HEC-1A), cervical (HeLa) and vaginal (VK2 E6/E7) cell lines; and, an *ex vivo* porcine vaginal model using the MTT reduction assay and histological analysis; having also demonstrated preoccupant toxicity profiles. In fact, Gino-Canesten® had one major impact on the vaginal *ex vivo* epithelia when histological analysis was performed (paper submitted for publication). Having this in mind, and concerning our cytotoxicity results, it was also herein demonstrated that these products could be considered *borderline* products, which means they have different classifications when using one scale or another. Moreover, Gino Travogen® and Colpotrophine®, were also the ones that presented higher toxicities on a toxicity *ex vivo* model that we developed. This data coincidence might corroborate the need to perform several *in vitro* tests for a complete toxicity evaluation, since the combined results might be stronger predictors for *in vivo* toxicity, as the current mind-set in toxicology states³⁵⁴. Furthermore, we also performed some technological characterization of the same products, having safety in perspective. Accordingly, we determined the osmolality, since it represents a predictor for irritation¹⁵³ and found that Lomexin® and Colpothrophine® were highly hyperosmolal (1446±20 and 1723±20 mOsmol/kg, respectively), above the upper limit recommended by the WHO (1200 mOsmol/kg)^{165,331}. In fact, isosmolality is well known to be an important technological parameter for safety of both ocular and vaginal products. Previously, we performed a similar study on vaginal lubricants and found that Geliofil®, Vidermina®, K-Y® Jelly, the ones that showed higher IS in the HET-CAM assay, had extremely high osmolalities (3582±11; 3707±16 and 3631±13 mOsmol/kg, respectively). Besides, Ginix and Ginix Plus were also the ones that showed higher toxic profiles on HeLa cervical cells using the lactate dehydrogenase (LDH) colorimetric cytotoxicity assay¹⁵⁹. Once again, K-Y® Jelly and Ginix Plus®, that were considered as HET-CAM *borderline* products, had previously demonstrated higher cytotoxicities. These findings, strengths the hypothesis that products osmolality and cellular and tissue toxicity could be highly predictive of irritation potential and, it can further suggest that these techniques (HET-CAM, osmolality, cell/tissue metabolic toxicity) should be applied concomitantly for a more robust clinical irritation response preview.

The application of the two scoring analytic methods, IS (A) and (B), confirmed that IS (B), although being more difficult to perform and also requiring a more qualified operator, can conduct to better classification output. Also, it leads, generally, to higher IS which means it could be scoring false positive irritants, rather than false negatives. In a safety perspective, this should be regarded as valuable in comparison to IS (A).

The suitability of the HET-CAM assay for vaginal irritation testing was demonstrated with this study, despite more assays and controls are needed to be enrolled to complete the validation process as well as inter-laboratory testing in order to confirm its reproducibility. Additionally, the improvements that were tested in the past for the HET-CAM applied to testing cosmetic ingredients eye toxicity, like an additional histological analysis³⁵⁵ and the combination of two softwares (ImageJ® and Adobe Photoshop®) that allows live monitoring of the assay, reducing the subjectivity in the endpoints evaluation³⁵⁶ shall be considered in this approach in order to assure more accurate results.

The HET-CAM assay has already been applied to test the irritation potential in other epithelia. In 1999, Lönnroth et al tested eight polymeric products to be used as dental restorative materials³⁵⁷. Later, on 2007 other research group inquired the irritative potential of dental adhesive agents, and answered this question by performing the HET-CAM assay³⁵⁸. Furthermore, it has been applied to testing of skin irritation, for the anti-inflammatory response of plasma to treat chronic skin wounds³⁵⁹, and also, to evaluate the irritation potential of topical antiseptics³⁶⁰. The HET-CAM has also been applied to test medicines for ocular application, for example compounded fluconazole and voriconazole eye drops prepared in an hospital pharmacy department to disclose potential eye irritation³⁶¹.

Although using incubated Hen's eggs for tests could represent a borderline case between *in vivo* and *in vitro* systems, it does not conflict with ethical and legal obligations especially animal protection laws. It was already demonstrated that incubation up to day nine, the embryonic differentiation of the chicken central nervous system is sufficiently incomplete to avoid suffering and pain perception. Actually, the few sensory fibers present at day nine only develop after incubation during 11 to 14 days¹⁹⁹. Studies also suggested that the extraembryonal vascular systems (e.g., yolk sac, CAM) are not sensitive to pain²⁰⁰. Therefore, this test method can reduce the number of animals subjected to testing and reduce the pain and suffering of rabbits by their exclusion from the testing of corrosives and severe irritants (U.S. Public Health Service, Department of Health and Human Services (2006)).

Until now no single *in vitro* test has emerged as being completely acceptable for full replacement of *in vivo* tests. However, the Hen's Egg Test Chorioallantoic Membrane has gained regulatory acceptance in various countries to classify severe eye irritants, and has potential to be applied to other mucosal/epithelial substrates such as the vaginal epithelia.

III.3.4. Conclusions

The HET-CAM assay was successfully applied to vaginal irritation testing. This strategy represents an innovative approach for the preclinical safety assessment of vaginal products, being them classified as medicines, cosmetics, hygiene products or medical devices. The application of the two scoring analytic methods, IS (A) and (B), confirmed that IS (B) can conduct to better classification output and preferably should be chosen. The studied vaginal formulations, comprising medicines and lubricants, showed, as expected, low potential for irritation. N-9 was considered as a severe irritant above 2% (w/v) concentrations, which corroborates clinical data from the literature, envisaging a possible *in vitro/in vivo* correlation. Comparisons with previous studies by our workgroup confirmed that HET-CAM can predict and/or confirm toxic profiles for products also tested for osmolality and cellular/tissue toxicity. Ideally, an integrative methodology should be designed to embrace all these preclinical tests, for a better *in vivo* safety preview. Although still requiring further validation, the HET-CAM assay seems an ideal prospect for vaginal irritancy potential *in vitro* studies.

CHAPTER IV

PRODUCT PERFORMANCE AND DRUG DELIVERY: *IN VITRO* RELEASE AND *EX VIVO* PERMEATION

The content of this chapter is partially published in:

Machado RM, Palmeira-de-Oliveira A, Ferreira AS, Borges L, Martinez-de-Oliveira J, Palmeira-de-Oliveira R, *“Vaginal drug delivery: in vitro release and ex vivo permeation of six active pharmaceutical ingredients from comercial semisolids, quantified by a sole HPLC-DAD validated method”*.

(submitted manuscript)

IV.1. GENERAL CONSIDERATIONS

The vaginal route of administration enables both local and systemic drug delivery^{4,5}. Safety and efficacy of drug delivery systems will rely on their ability to promote adequate drug concentrations at the targeted site of action, being it located on the vaginal epithelia itself, or in a distant location. Two crucial steps contribute to this achievement: first, the drug release from the formulation, and, second, the ability to permeate the biological barrier¹⁷.

Drug release, or *in vitro* drug release, is one of the standard methods that can be used to characterize the performance of a finished topical dosage form, i.e., semisolids, such as creams, gels, and ointments. It could represent a valuable tool for both the initial formulation design but also as quality control step, since important changes in the characteristics of a drug product formula can result in differences regarding drug release rate^{251,262}.

Furthermore, studies on drug permeation through biological barriers are important to infer on the ability of the drug to exert systemic effects. In the context of local therapy these studies are important for safety characterization since the objective is to limit the activity to the vaginal wall, surface or contents. On the other hand, when a systemic effect is desired, permeation studies are mandatory. The *in vitro* models developed to predict drug permeation not only provide preliminary information on absorption rates and efficacy, but also help investigating and understanding the pathogenesis of various microbiological diseases^{208,362,363}. So, applicable and reproducible methods are important to provide comprehensiveness about mechanisms of permeation, absorption and mode of action of active substances for vaginal application³⁴ and also to characterize vaginal drug delivery systems³⁹. The *ex vivo* porcine vaginal model has already been described for vaginal permeation studies^{34,364}.

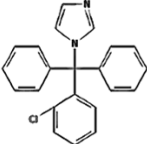
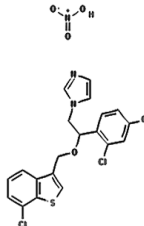
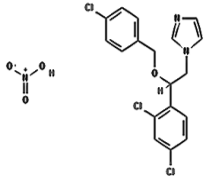
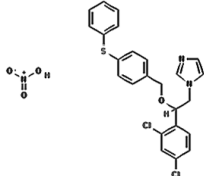
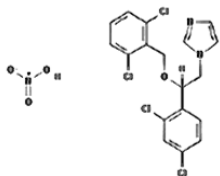
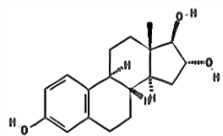
IV.1.1. Objectives

The aims of the study comprised in this chapter are to:

- Fully validate a HPLC-DAD method for the quantification of six active pharmaceutical ingredients (API) (Table IV.1) present in a representative group of vaginal semisolid formulations, commercialized internationally;
- Disclose the *in vitro* release and *ex vivo* permeation of these drugs through experimental settings that consider the particularities of the vaginal environment.

For each one of these techniques, a partial validation of the quantification method was performed. Release and permeation studies were accomplished in static vertical Franz diffusion cells, and concerning the latest, the porcine model was applied through a technique previously developed by our workgroup.

Table IV. 1: Chemical characteristics of the drugs included in this study. Adpated from PubChem³⁶⁵, Drug Bank³⁶⁶, and ChEMBL from the European Bioinformatics Institute³⁶⁷.

Molecule	Structure	MW (g/mol)	logP	pKa
Clotrimazole (CLT) $C_{22}H_{17}ClN_2$ CAS number: 23593-75-1		344.837	6.1	6.6
Sertaconazole Nitrate (SER) $C_{20}H_{16}Cl_3N_3O_4S$ CAS number: 99592-39-9		500.775	6.2	6.8
Econazole Nitrate (ECO) $C_{18}H_{16}Cl_3N_3O_4$ CAS number: 24169-02-6		444.693	5.4	6.8
Fenticonazole Nitrate (FEN) $C_{24}H_{21}Cl_2N_3O_4S$ CAS number: 73151-29-8		518.409	6.4	6.7
Isoconazole Nitrate (ISO) $C_{18}H_{15}Cl_4N_3O_4$ CAS number: 24168-96-5		479.135	6.0	6.8
Estriol (EST) $C_{18}H_{24}O_3$ CAS number: 50-27-1		288.387	2.5	10.5

IV.2. EXPERIMENTAL

IV.2.1. Materials and Methods

IV.2.1.1. Chemicals

Clotrimazole (CLT - TCI, Japan), sertaconazole nitrate (SER - EDQM, France), econazole nitrate (ECO - TCI, Japan), fenticonazole nitrate (FEN - TCI, Japan), isoconazole nitrate (ISO - TCI, Japan) and estriol (EST - TCI, Japan) were purchased as analytical standards for HPLC. Methanol (Fisher Chemical, United Kingdom) was of HPLC-grade. Polyethylene glycol 400 (PEG 400, Merck, Germany), propylene glycol (Acros, USA), poloxamer 407 Pluracare® F127 Prill (BASF, Germany, ethanol (Aga, Portugal), dipotassium hydrogen phosphate (VWR Prolabo, USA), phosphoric acid (VWR Prolabo, USA) and sodium chloride (JT Baker, United States of America) were of analytical grade. Methanol (Fisher Scientific, United Kingdom) was HPLC gradient grade, an ultrapure water system (Millipore Corporation, USA) was used to purify water to 18.2 MΩ.cm². All other chemicals and solvents were of analytical grade or equivalent.

IV.2.1.2. Testing formulations

The semisolid products tested in this study are classified as medicines and were acquired in Portuguese community pharmacies, being also commercialized across the EU and USA. Namely, they were: Gino-Canesten®, Sertopic®, Dermofix®, Gyno-Pevaryl®, Lomexin®, Gino Travogen®, Ovestin® and Blissel®. Specific characteristics of these products are listed in **Table II. 1**.

IV.2.1.3. Quantification method

IV.2.1.3.1. Instrument and chromatographic conditions

Drugs were quantified using a High Pressure Liquid Chromatograph (HPLC, LC-2010AHT Shimadzu, Japan) equipped with a Diode Array Detector (SPD-M20A) set at 210 nm. All instrumental components were controlled by Labsolutions software (Shimadzu, Japan). Method optimization and development was achieved by consulting the molecules' chemical characteristics (**Table IV.1**). The mobile phase was methanol:25 mM dipotassium hydrogen phosphate pH 7.5 (75:25, %v/v) set at a 1.5 mL/min flow rate. The injection volume was 20 µL. An MZ ZORBAX ODS-C18 (4.6 x 250 mm, 5 µm) column at 30°C was used for analytical separation. A Phenomenex C18 security cartridge (4 x 3 mm) was attached before the column. Peak areas were used for quantifications. The aqueous mobile phase was filtered through a 0.22 µm filter (Nyalaflo, PALL Life Sciences, USA) and degassed by ultrasound during 10 minutes before use.

IV.2.1.3.2. Preparation of stock solutions, calibration standards and quality control samples

A stock solution for each individual molecule was prepared in methanol (1 mg/mL). Subsequently, working standard solutions (100 and 300 µg/mL) were obtained through stock

standard dilution. A combined solution of all the drugs was prepared at 1 mg/mL and 20 µg/mL. Then, eight standard solutions (250; 100; 50; 25, 10; 5; 2 and 1 µg/mL) were obtained by independent dilutions from the working standard mixtures in methanol (for the main method), and in the receptor media used for drug release and permeation (for partial validations). The stock, working and combining solutions were stored under refrigeration (4°C) and protected from light. Quality control (QC) samples, were prepared from independent stock solutions, to represent four concentration levels: QC1, at the lower limit of quantification (LLOQ) of the respective drug; QC2, at three times de LLOQ; QC3, at an intermediate concentration - 125 µg/mL; and QC4 at a high level - 200 µg/mL.

IV.2.1.3.3. Method validation

The international recommendations for bioanalytical method validation from Food and Drug Administration (FDA, 2011), European Medicines Agency (EMA, 2012) and International Conference for Harmonization (ICH, 2005) were followed to validate the method herein developed^{270,368,369}. Accordingly, the following parameters were considered: selectivity, linearity, precision, accuracy and limit of quantification. A full validation was performed for the standards in methanol (three independent days - between-run - with a daily calibration curve and a set of QCs, and a day of within-run study with a daily curve and a set QCs, n=5), and partial validations were performed for the receptor media selected for the drug release and drug permeation experiments (daily calibration curve and a set of QCs, n=3).

Selectivity

Selectivity was studied by running different blank samples, including the final matrices (receptor media), and by comparing these chromatograms with the ones obtained for drug samples.

Linearity

Linearity was determined by calculating a regression line from the peak area vs. nominal concentration plot for the standard solutions (250; 100; 50; 25; 10; 5; 2 and 1 µg/mL) using linear least squares methodology, calculation of the correlation coefficient (r^2), back calculation of the concentration of the individual calibration standards and by analysis of the respective response factors.

Precision and Accuracy

Method inter-batch and intra-batch precision and accuracy was accessed by analysing QC samples on three consecutive days at the four concentration levels (QC1-4), representative of the calibration range. Furthermore, the intra-batch precision and accuracy were assessed analysing five sets of the QC samples in a single day. Taking into account the acceptance criteria defined by the bioanalytical method validation guidelines, the intra- and inter-batch precision

(expressed as percentage of CV) must be $\leq 15\%$ (or 20% in the LLOQ) and intra- and inter-batch accuracy (expressed as percentage of bias) must be within $\pm 15\%$ (or $\pm 20\%$ in the LLOQ).

Limits of quantification (LLOQ)

The lower limit of quantification (LLOQ) is defined as the lowest concentration of the calibration curve, which can be quantified with adequate inter/intra-batch precision and accuracy. The precision, expressed as percentage of coefficient of variation (%CV), cannot exceed 20% ; whereas accuracy, expressed by the deviation from nominal concentration value (bias), must be within $\pm 20\%$.

Stability

Drugs stability of QCs 2 and 4 was assessed at 24 h and 7 days when stored at 4°C .

IV.2.1.4. Solubility testing

Solubility studies were carried out for all drugs to determine the solubility in different possible receptor media. Receptor media for both *in vitro* release and *ex vivo* permeation studies were chosen taking into consideration the drug solubility and respecting the need for maintaining *sink* conditions all over the assays. For *sink* conditions maintenance, it is accepted that the solvent volume shall be 10 times greater than the volume of the saturated solution of the targeted chemical contained in the dosage form to be tested. Thus during release and permeation testing, *sink* conditions are mandatory, otherwise the drug concentration could get close to the saturation point, limiting the dissolution rate^{370,371}. The method performed was adapted from Gupta *et al.* (2011)³⁷².

For each media two pH were tested: 4.2 and 7.4, respectively for use in the *in vitro* release and *ex vivo* permeation studies. The pH of 4.2 represents the vaginal pH of healthy reproductive aged women, thus representing the pH condition for drug release. The pH of 7.4 represents the systemic circulation, so it was used in the permeation experiments. The receptor media tested were: 40% ethanol:60% PBS; 0.5% poloxamer:PBS; 1% poloxamer:PBS and PBS. For CLT, 40% propylene glycol:60% PBS; 60% propylene glycol:40% PBS and 40% PEG 400:60% PBS were evaluated. Solubility testing in these media was performed by suspending a drug amount (~ 1 mg/mL) that was calculated as high enough to respect *sink* conditions (experimental setup calculations for predictive concentrations on the receptor chamber after permeation experiments are available on APPENDIX D). Samples were incubated in a 37°C water bath at 80 RPM during 24 hours. After 24 hours, the samples were centrifuged at 10.000 RPM for 10 minutes. The supernatants were collected and filtered through $0.22\ \mu\text{m}$ PTFE filters (VWR, USA) and injected into HPLC for analysis.

IV.2.1.5. *In vitro* drug release

The *in vitro* drug release studies were performed in Static Franz Diffusion Vertical Cells (PermeGear, GmbH Analysesysteme, Germany). The receptor chamber was filled with the respective media up to 15 mL and magnetic stirring was maintained at 500 RPM. The contact area was 0.64 cm² and on the donor chamber app. 300mg of each formulation were applied (weighted on an analytical balance on pre-filled syringes, before and after application on the chamber to determine the exact amount dispensed and available for diffusion). The interface between the two chambers was an inert membrane (Supor®-450, PALL Life Sciences, USA) and the total experiment time was 8 h, with samples (200 µL) being collected every 30 min. The volume collected was replaced by pre-heated fresh media. Samples were immediately quantified by HPLC-DAD. The amount of drug released per unit area (µg/cm²) was plotted against the square root of time with the slope representing the release rate.

IV.2.1.6. *Ex vivo* drug permeation

Ex vivo drug permeation experiments were performed not only to disclose the drug profile of permeation but also to compare caudal and cranial porcine vaginal epithelium permeability. The porcine vaginal tubes were kindly provided by a local slaughterhouse and processed in the lab within 4 h after the sacrifice. The tubes were incised longitudinally in order to expose the vaginal epithelium. Then, the tissues were washed in HBSS at pH 7.4, pre-warmed at 37°C, wrapped individually in aluminium foil and packed in plastic bags to be frozen at -20°C for up to 6 months.

Before the permeation study, tissues were thawed in a saline solution (NaCl 0.9%) at room temperature. After defrosting, they were manually dermatomed in order to standardize tissue thickness to 300-400 µm (BBraun equipped with Aesculap blades, Germany). Tissue thickness was confirmed using a micrometer (Vogel, Germany). Tissue diameter was adjusted using a surgical scalp in order to fit the Franz cell diameter. Specimens from the caudal and cranial vagina were tested to assess differences between the two in terms of drug permeation (n=3 for each one, 3 different animals). A Static Franz Diffusion Vertical Cell equipment (PermeGear, GmbH Analysesysteme, Germany) was used to assembly 6 cells in the same experimental set. The experiment was extended for 48 h, and 200 µL aliquots were collected every 4 h being immediately replaced by fresh receptor media pre-heated at 37°C. These collected aliquots were directly analysed by HPLC-DAD. At the end of the experiment, the donor chamber was extracted with 25 mL of methanol and diluted ten times in methanol. Tissues were also collected and extracted by a 30 min sonication with 2 mL of methanol. These samples were filtered with PTFE pore 0.45 µm, before analytical quantification. Results obtained from this quantification were used to calculate the mass balance according to the OCDE guideline, considering the total amount of drug recovered (from the receptor compartment, the tissue and the donor compartment) and the initial amount of drug placed in the donor compartment, which should be within 100% ± 20%.

Tissue integrity after the permeation experiment was confirmed by histology and through a trypan blue based technique³⁷³. Therefore, control tissues (blank experiments), n=4 per tissue type (caudal and cranial), were left in Franz cell with no formulation during the experimental time defined, 48 h. After that time, one tissue was collected and fixed in a balanced 10% formalin solution and stained with haematoxylin and eosin. Slides were observed and microphotographs were taken using a Zeiss microscope (Axiomager A1, Zeiss, Germany) equipped with a digital camera (AxioCam, Zeiss, Germany). The remaining tissues were used for integrity validation with trypan blue. 100 µL of a trypan blue stock solution (0.3 mg/mL) were applied on the apical side of the membrane and let to permeate. After 15 min, apical and basolateral solutions (i.e. donor and receptor solutions) were collected and absorbances read at 590 nm. Receptor solution without the application of trypan blue was used as a control.

The apparent permeability coefficient (P_{app}) and steady-state flux (J_{ss}) were calculated based on the Fick's First Law of Diffusion and were used as equators between the two anatomically different epithelia. P_{app} was calculated from the drug levels measured in the receptor compartment according to the equation: $P_{app}(cm.s^{-1}) = \frac{Q}{A \cdot t}$. Where Q is the total amount of permeated drug (µg), A is the diffusion area (cm²), C the initial concentration of drug in the donor compartment (µg/cm³), and t the total time of the experiment (s). Additionally, the coefficient Q/At represents the steady-state flux (J_{ss}) of drug across the epithelial layer³⁷⁴. Graphical representations of the amount of drug that permeated both membranes were plotted as µg/cm² vs h.

IV.2.1.7. Data processing and statistical analysis

Data was analysed to produce arithmetic means with standard deviations (SD) using Microsoft Excel. Linear regression was also accomplished with Excel. Analysis of variance (ANOVA) was performed to determine the significance of the difference between sets of data ($p < 0.05$) using GraphPad Prism 6.0 (Graph Pad Software, USA).

IV.2.2. Results and Discussion

IV.2.2.1. Quantification method validation

The analytical separation of CLT, ECO, EST, FEN, ISO and SER was successfully achieved using the chromatographic conditions previously described. These analytical conditions conducted to a total run time of 50 minutes. However, on the assays where the method was to be applied (drug release and drug permeation) the run time was adjusted to each molecule. The compounds elution order was: EST, CLT, ECO, ISO, SER and FEN. A representative chromatogram of a standard run (50 µg/mL) is shown on **Figure IV. 1**. A good resolution between analytical peaks was obtained. Also, peak shapes were acceptable, even on the partial validations (data not shown). Calibration parameters obtained for the three analytical methods (one full validation and two partial validations) are described on **Table IV.2**.

Actually, we performed the full validation method, in which the molecules were directly dissolved in methanol, and considered this as our standard method because we had several studies to perform within this media (methanol), such as the stability testing, the drug recovery testing from the semisolids and further, the receptor chambers and tissues resulted from the permeation experiments. Since this analytical method has showed to be highly reliable in the full validation process, we proceeded with partial validation for the remaining matrices used on the *in vitro* release studies and *ex vivo* permeation. As shown through the calibration parameters (**Table IV. 2**) we were able to achieve linear behaviours for all analyses in the 3 medias ($r^2 \geq 0.9943$, generally over 0.98 is well accepted by regulatory authorities) in the ranges defined. Although some ranges might seem wide, it is important to bear in mind that the method shall allow for the quantification of either small concentrations in samples from *in vitro* and *ex vivo* studies, and high concentrations obtained from product extractions. The LLOQs, which correspond to QC1s, were experimentally defined and its accuracy and precision were in accordance to the 20% acceptable variation from their nominal concentrations²⁶⁹. Furthermore, the others QCs maintained these parameters bellow 15%.

Table IV. 2: Calibration parameters for CLT, ECO, EST, FEN, ISO and SER after the full validation and partial validations for the receptor media used on *in vitro* drug release and *ex vivo* permeation studies.

	Analyte	Calibration parameters		
		Range (µg/mL)	Equation	r^2
Full validation	CLT	1 - 250	$y=84806x-730.95$	1.0000
	ECO	2 - 250	$y=36948x+28233$	0.9999
	EST	1 - 250	$y=2757.4x-15135$	0.9949
	FEN	10 - 250	$y=52748x-50206$	0.9999
	ISO	2 - 250	$y=39971x-56071$	0.9997
	SER	5 - 250	$y=59677x-97810$	0.9997
Partial validation for <i>in vitro</i> drug release	CLT	1 - 250	$y=52362x-6508$	0.9991
	ECO	2 - 250	$y=102318x+423692$	0.9969
	EST	1 - 250	$y=29896x-17291$	0.9996
	FEN	10 - 250	$y=17590x+2277$	1.0000
	ISO	2 - 250	$y=50167x-17933$	0.9999
	SER	5 - 250	$y=43400x+75517$	0.9998
Partial validation for <i>ex vivo</i> drug permeation	CLT	1 - 250	$y=34201x-41662$	0.9998
	ECO	2 - 250	$y=48102x-35665$	0.9994
	EST	1 - 250	$y=27449x-60359$	0.9962
	FEN	10 - 250	$y=35977x+522218$	0.9943
	ISO	2 - 250	$y=53009x-66707$	0.9998
	SER	5 - 250	$y=40647x+61121$	0.9997

Complete data from within- and between-run precision and accuracy is detailed in **Table IV.4**. For the full validation process a complete assessment of concentration levels (QC1-QC4) was performed, while for the partial validations only QC2 and QC4 were analysed. All the data fulfilled the acceptance criteria established. The drugs stability was studied as described on the “Methods” section. The same vial (QC) was quantified just after preparation, after 24 hours and after 7 days. Storage was performed at 4°C protected from light which is the expected storage period for samples before quantification (**Table IV.3**).

Table IV. 3: Stability of CLT, ECO, EST, FEN, ISO and SER in a methanolic solution considered in the full validation process. Results are presented as percentual variation from t_0 samples.

Drug Stability			
Analyte	Nominal concentration (µg/mL)	t_{24h} (%)	t_{7d} (%)
CLT	3	-7.1	10.5
	200	-7.7	11.2
ECO	6	3.6	12.4
	200	-8.0	2.9
EST	3	8.7	-3.7
	200	-11.3	2.7
FEN	30	4.8	10.7
	200	3.3	3.5
ISO	6	-6.7	-9.5
	200	5.8	14.0
SER	15	14.3	0.2
	200	-7.1	-4.2

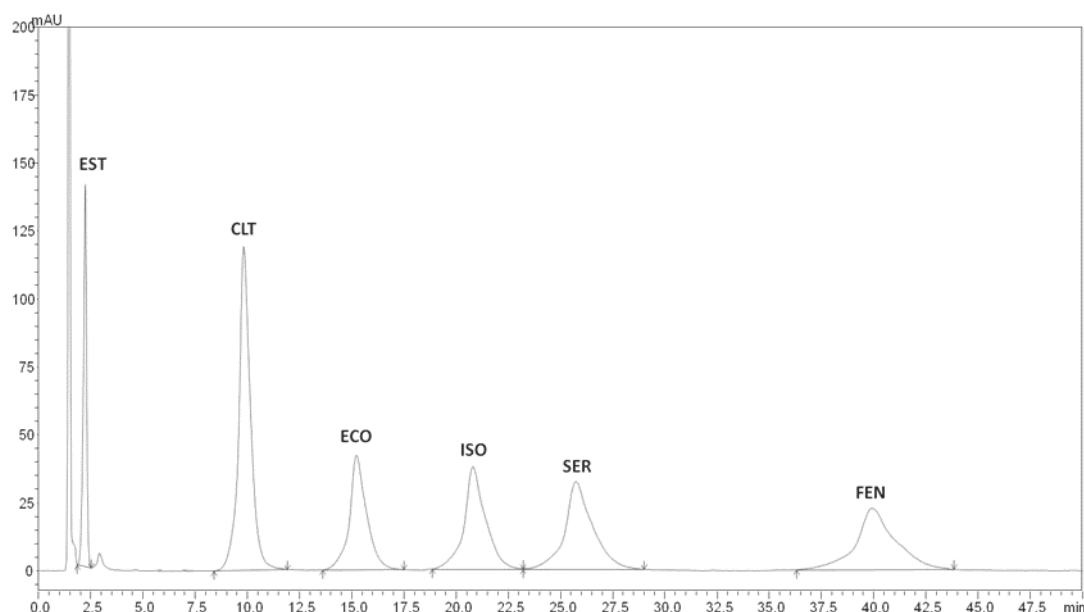


Figure IV. 1: Representative chromatogram of the quantification method in the full validation process, HPLC-DAD, measured at 210 nm (standard sample from the calibration curve at 50 µg/ml). The six molecules were identified and quantified as separated peaks in a single run.

To our best knowledge, the analytical method herein presented is the first one to include five antifungal molecules and an oestrogen. These are the most representative APIs included in currently marketed vaginal semisolids. Although in this study these APIs were quantified alone in each drug release or permeation study, this method allows for further applications such as the development of new products containing combinations of these drugs. Indeed, despite the topical treatment of vaginal infections has been shown to be effective in most cases, the microbial resistance to chemotherapeutics and the difficulties in managing infection recurrences sustain the need for more effective local treatments¹³². Actually, there is a continuous need for new analytical methodologies that shall be quicker, more sensitive, accurate and precise, that allow for drug quantification in biological samples, and that also could support the various stages of drug discovery and development^{375,376}. Our technique was easily translated to biological simulants, showing that it is promising for application in, for example, cell culture medias (*in vitro*) and even biological samples collected on *in vivo* studies. Actually, the complexity and quality of bioanalytical assays increases with the stage of drug/product development. Moreover, the validation criteria from international guidelines are not usually fully applied in the early stages of drug discovery and development^{375,376}. This could be the reason why there are few HPLC validated methodologies described on the scientific literature regarding *in vitro* release and *ex vivo* permeation²⁸². This work shows a robust technique that is able to provide results with a high reliability and that complies with the international standards from the US Food and Drug Administration and the European Medicines Agency^{270,368}.

Table IV. 4: Within and Between Run precision (% CV) and accuracy (% bias) obtained for CLT, ECO, EST, FEN, ISO and SER at concentrations of the Lower Limit of Quantification (QC1) and at the low (QC2), middle (QC3) and high (QC4) concentrations representative of the calibration range.

Full validation						Partial validation - <i>in vitro</i> release					Partial validation - <i>ex vivo</i> permeation			
Analyte	Nominal conc. (µg/mL)	Within-run		Between-run		Nominal conc. (µg/mL)	Within-run		Between-run		Within-run		Between-run	
		Precision (% CV)	Accuracy (% bias)	Precision (% CV)	Accuracy (% bias)		Precision (% CV)	Accuracy (% bias)	Precision (% CV)	Accuracy (% bias)	Precision (% CV)	Accuracy (% bias)	Precision (% CV)	Accuracy (% bias)
CLT	1	5.1	-11.4	0.9	-16.0	3 200	4.1 0.3	1.3 6.8	6.3 1.4	2.8 0.6	1.8 5.7	12.1 -2.8	7.8 8.3	2.7 -0.6
	3	7.7	-1.3	2.2	5.7									
	125	3.9	-4.0	9.5	-0.2									
	200	4.1	7.3	2.1	6.3									
ECO	2	4.7	-19.4	5.4	-18.0	6 200	5.8 12.2	8.3 4.7	8.6 6.1	-5.4 -0.3	8.2 5.1	-2.0 -5.0	0.2 1.4	3.3 -10.8
	6	8.0	2.0	15.0	-1.0									
	125	5.2	5.7	10.8	-2.4									
	200	3.1	9.8	5.4	5.9									
EST	1	4.1	10.2	3.0	7.6	3 200	3.2 4.6	2.5 1.5	3.9 9.8	-2.7 -2.9	4.8 3.4	8.9 -7.5	0.3 2.0	5.5 7.9
	3	6.7	-0.9	4.8	7.7									
	125	11.8	2.8	14.4	3.4									
	200	2.4	-2.1	6.1	1.6									
FEN	10	6.9	-3.7	10.5	5.5	30 200	1.7 6.1	12.5 -4.0	1.6 0.5	3.8 -1.2	3.7 6.0	5.4 7.6	4.1 4.6	0.3 7.0
	30	7.4	2.4	9.2	-4.7									
	125	6.3	3.9	11.8	1.9									
	200	4.7	5.0	5.5	5.5									
ISO	2	3.2	6.4	2.4	9.2	6 200	6.3 1.6	-10.6 4.9	0.1 0.6	-12.0 -5.2	5.2 6.0	5.7 -2.5	8.3 0.6	1.4 4.9
	6	11.2	2.7	9.9	-3.5									
	125	6.5	5.4	8.3	-0.7									
	200	1.7	8.6	2.5	8.2									
SER	5	8.5	-5.6	13.4	-7.0	15 200	4.3 2.1	8.5 -0.7	9.0 4.9	-4.7 -0.4	1.8 2.9	2.5 9.3	9.0 7.5	-1.1 1.8
	15	10.3	1.9	5.9	-8.9									
	125	4.8	5.8	11.6	1.3									
	200	5.5	5.7	5.7	3.3									

IV.2.2.2. Drug solubility

Drug solubility was determined to assure that *sink* conditions could be maintained during the assays of drug release and drug permeation³⁷⁷. Therefore, we should obtain high solubility in the simplest media, that also could, in some extent, include some physiologic featuring's. A phosphate buffered solution was thought as being the most representative. However, this solution had to be mixed with other solvents to increase drugs solubility. Then, the pHs were differentiated for *in vitro* release and *ex vivo* permeation, since the first is expected to occur in the vaginal cavity, where the normal pH is 4.2, and the second is expected to occur over the vaginal epithelia where the interface to the systemic circulation is present. **Table IV. 5** demonstrates the solubility obtained for the drugs included in this study. CLT was studied in different media because it was difficult to solubilize in the first ones (data not shown). For *in vitro* release studies the media selected were a mixture of ethanol and phosphate buffer solution (EtOH:PBS, 40:60) at pH 4.2 for drugs ECO, EST, FEN, ISO and SER. CLT *in vitro* release was performed using PEG 400 : PBS (40:60) at pH 4.2. All *ex vivo* permeation experiments required the use of EtOH:PBS (40:60) at pH 7.4, except CLT which was performed with a media containing PEG 400.

Table IV. 5: Solubility ($\mu\text{g/mL}$) for CLT, ECO, EST, FEN, ISO and SER in solutions tested as possible receptor media for *in vitro* drug release and *ex vivo* permeation studies. The receptor media selected to be used in further experiments were marked with *.

Drug	Drug solubility ($\mu\text{g/mL}$)					
	PBS	pH 4.2 EtOH:PBS (40:60)	PLX 0.2%:PBS	PBS	pH 7.4 EtOH:PBS (40:60)	PLX 0.2%:PBS
ECO	386.1	967.5*	664.6	13.1	561.0*	75.7
EST	37.2	>1000*	44.4	39.7	>1000*	45.9
FEN	<10 $\mu\text{g/mL}$ (LLOQ)	789.7*	93.7	<10 $\mu\text{g/mL}$ (LLOQ)	223.6*	10.3
ISO	178.5	971.5*	68.2	14.3	>1000*	22.0
SER	19.9	784.5*	30.8	<5 $\mu\text{g/mL}$ (LLOQ)	659.0*	59.8
CLT	PBS	pH 4.2 PEG 400:PBS (40:60)	PLX 0.5%:PBS	PBS	pH 7.4 PEG400:PBS (40:60)	PLX 0.5%:PBS
	17.0	282.1*	0.7	0.6	764.8*	4.8

This study was essential to assure that molecules would have enough solubility in receptor media to be able to diffuse and to cross the membranes (inert or biological) and achieve the receptor chamber. Actually, this was a limitation found during literature review, since data on drug solubility is frequently not reported in published studies even in those that conclude that the drugs did not transpose the membranes. On the contrary, several other authors addressed this issue^{139,282,283,372} although, there was no sufficient data available regarding solubility of the drugs included in this study. Therefore, this full assessment of solubility was mandatory for this study.

IV.2.2.3. *In vitro* drug release

Concerning *in vitro* drug release studies, SER (from Sertopic® and Dermofix®) was not able to be released on the experimental setting conducted in this study. In fact, the analyte was not quantifiable after the 8 h experiments in none of the Franz cell (n=6). Despite having an acceptable solubility in the receptor media that was chosen, this molecule seems to have higher affinity with the formulation than with receptor media. On our permeation experiments, however, SER was quantifiable which leads us to hypothesize that drug release from the formulation might be related with the media pH and, consequently, drug ionization factors. In fact, *in vitro* drug release was accomplished for SER from a microemulsion hydrogel in a 10% (w/v) HBCD ((2-Hydroxypropyl)- β -cyclodextrin)-solution³⁷⁸; from bioadhesive gels in a phosphate solution with pH 6.8³⁷⁹; from an anhydrous gel in a PEG 400:PBS pH 7.4 solution³⁸⁰; and, from microsponges in a phosphate buffer pH 6.8:methanol solution³⁸¹. Nonetheless, some of these studies were not clear on naming the experiments and attributed away the term “*in vitro* drug release” to experiments in which biological surrogates were used as membranes. Additionally, these different formulation approaches for SER being more aqueous-based could circumvent the release limiting step in oil-containing formulations and further increase release^{382,378}. The remaining *in vitro* drug release profiles are represented in **Figure IV. 2**, and information concerning the diffusion rates are detailed in **Table IV. 6**.

Regarding EST, the total amount released from Ovestin® and Blissel® was quite different, although the diffusion rate was similar (20.90 and 13.34 $\mu\text{g}/\text{cm}^2/\text{h}$, with r^2 0.9977 and 0.9602, respectively). Despite Ovestin® showed a slightly higher release rate, the total released amount was higher for Blissel® (57.20 \pm 10.93 and 48.16 \pm 4.05 $\mu\text{g}/\text{cm}^2$). This was an interesting finding, that highlighted the easiness of EST release from hydrophilic formulations, since in fact the EST concentration in both formulations were very distinct (Ovestin® 1 mg/g and Blissel® 50 $\mu\text{g}/\text{g}$). EST *in vitro* release studies have already been performed in a PBS-hydroxypropyl- β -cyclodextrin (HPBCD) media, being the drug solubility in the receptor media nearly half than the one reported in this study (560.40 $\mu\text{g}/\text{mL}$), leading to a released amount after 8 h approximately half than the herein reported (25 $\mu\text{g}/\text{cm}^2$)³⁸³. This reinforces the need to present solubility data when performing these kind of studies, so results are comparable between workgroups. FEN had the higher diffusion rate of all drugs (4805.5 $\mu\text{g}/\text{cm}^2/\text{h}$), but it is also a higher concentrated formulation in terms of active substance (20 mg/g). Drug release for Gino Travogen® occurred just after 30 min, being the first to start releasing its active substance. Gino-Canesten® only started to release the API (CLT) after 2 h. In release studies it is expected that a lag time occurs, as an X intercept corresponding normally to a fraction of an hour, reflecting the time required for the diffusion process to reach the equilibrium²⁵¹. The antifungal azole molecules FEN, ISO and CLT are comparable considering our results. In fact, these formulations are expected to release their drugs within eight hours since the vaginal formulations are usually recommended to be applied, at night, before sleeping. During this time, the drug is expected to be released, so it can exert its therapeutic action. However, a quick and extensive release

can also trigger side effects, and azolic antifungals are well-known by their concentration-dependent toxicity²⁸¹.

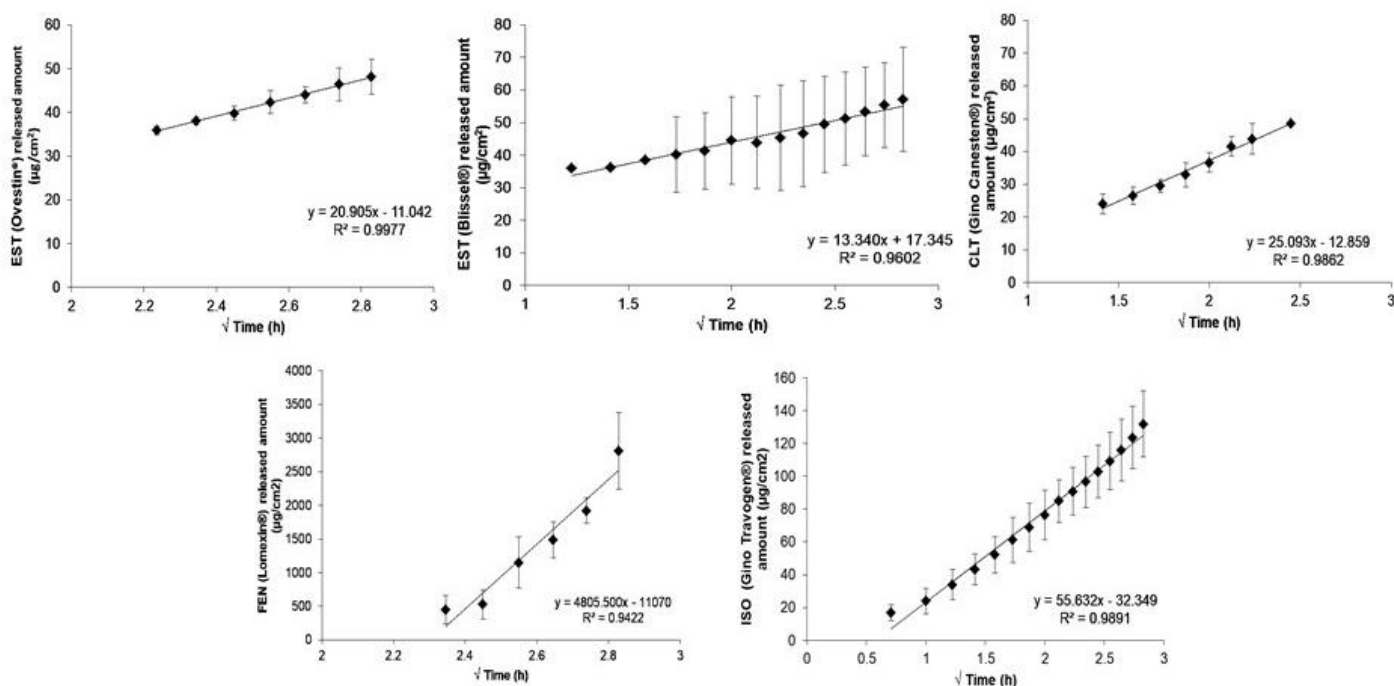


Figure IV. 2: *in vitro* drug release (µg/cm²) profiles of Ovestin® (EST), Blissel® (EST), Gino-Canesten® (CLT), Lomexin® (FEN) and Gino Travogen® (ISO) as function of square root of time (h). The release rate was inferred by the slope obtained for each profile. Results are means and bars represent standard deviations (n=6).

It was our option to conduct the fully assessment of *in vitro* release during 8 h and to present the total profiles instead of limited points for tracing the linear profile, as recommended by the FDA-SUPAC document (Scale-up and Post-Approval Change)²⁵¹. Actually, it was our intention to see the complete release profiles, since what we are evaluating is the correspondence of release and our proposed *ex vivo* permeation method. Although working on an extended time period, at the end of the experiments drug content in the receptor chamber did not exceeded 30% of the amount placed on the donor chamber, which would indicate excessive depletion of drug from the donor compartment and would be translated as a curvature in the upper part of the plots. These observations confirm that the experimental setting corresponded to an “infinite dose”.

IV.2.2.4. *Ex vivo* drug permeation

Ex vivo drug permeation was tested using a porcine vaginal *ex vivo* model. Porcine vaginal epithelium *ex vivo* has been chosen for membrane model in vaginal permeation experiments since the pig has been already cited as remarkably similar with human in terms of anatomy, physiology, metabolism and histology. Furthermore, previous studies have reported that an excellent correlation was found between human and porcine vaginal tissues^{34,237,246,364}.

However, our group has found that tissues collected from a caudal or cranial region in the vagina have different histological arrangements (submitted for publication). Considering this, tissues from two different anatomical regions were collected (caudal and cranial vagina). Tissues integrity had to be assured along the experiments time, and histological analysis confirmed that this *ex vivo* model could provide tissue stability until 48 h of a permeation experiment **Figure IV. 3**. Actually, cranial vagina seems to better maintain its epithelial structure after the experiment. However, caudal vagina has a thicker epithelial vagina that is more exposed to external factors, since in the cranial tissue most of this thin epithelial layer is “protected” by the *ruggae*. These *ruggae per se*, also contribute to a larger variability in the tissues surfaces, regarding the permeation experiments, where a uniform layer is aimed in order to obtain less data variability. Furthermore, trypan blue was used to assure the tissue was still impermeable to hydrophilic solution after the 48 h experiments. In this assay, no statistical difference was found between the blank solution absorbance (receptor media not tested with trypan blue) and the medias obtained from chambers where the trypan blue test was performed (One-way ANOVA, $p < 0.05$).

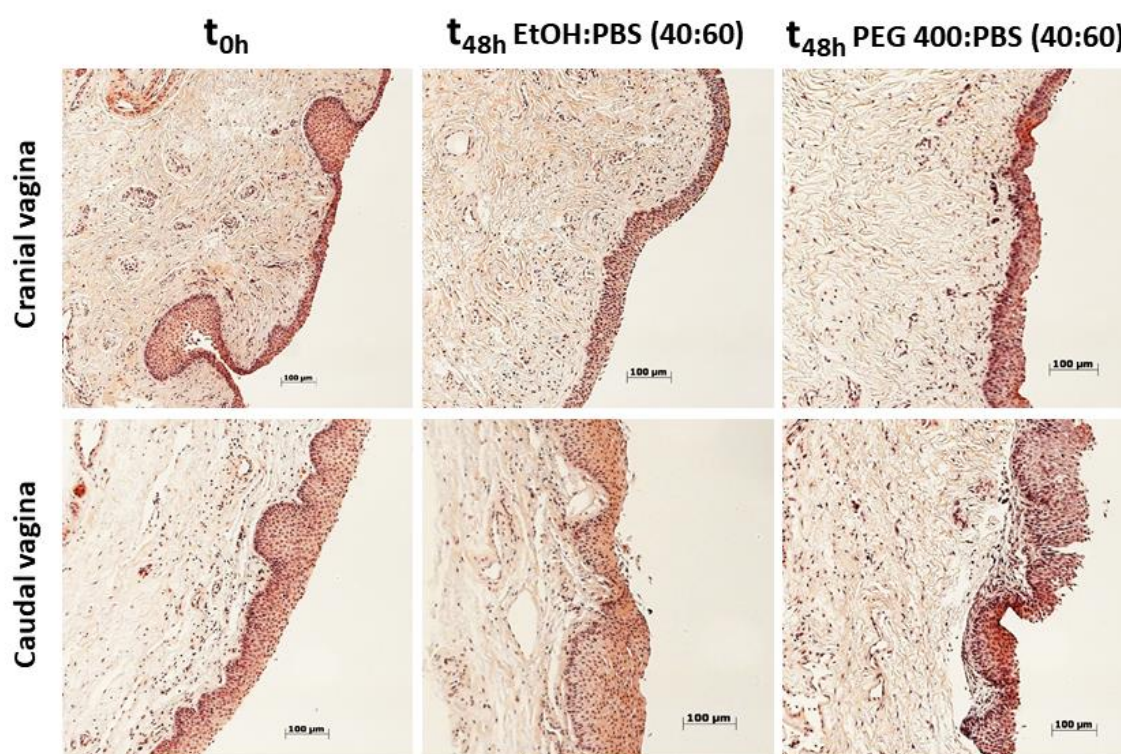


Figure IV. 3: Histological images of the porcine *ex vivo* model used in the permeation experiments. Tissues collected at the end of the experiments (t_{48h}) where no formulation was applied are shown of the middle and right column. The left column shows specimens right-after tissue preparation (t_{0h}).

Tissue thickness was determined by means of micrometer after epithelial sheets excision, and it was confirmed that our tissue collecting procedure can conduct to homogeneous tissues. Thickness was determined for all animals used in the experiments, in triplicate. For cranial vagina, it was numerically $390 \pm 68 \mu\text{m}$, which represents a total variation coefficient of 17.4%.

While caudal vagina had a thickness of $327 \pm 73 \mu\text{m}$ (CV of 22.5%). The *t*-test ($p < 0.05$) proved that the thickness difference between these two tissues is statistically supported. Despite caudal vaginal had a higher CV, which however is within acceptable limits ($<25\%$), it is also demonstrated that this surrogate has significant lower thickness when compared with cranial vagina, and is more in the range that was expected for permeation studies (200-400 μm).

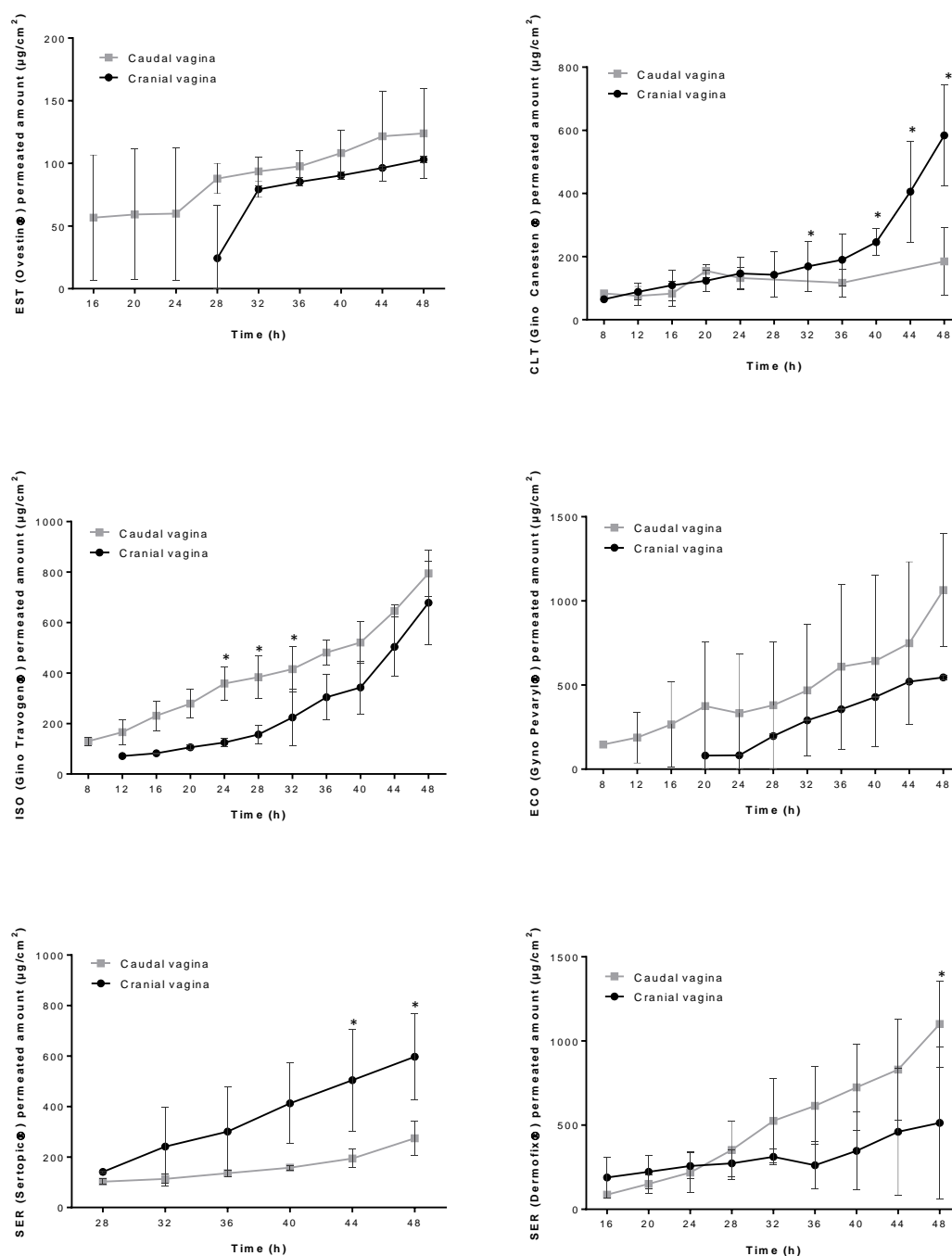


Figure IV. 4: ex vivo permeation ($\mu\text{g}/\text{cm}^2$) profiles of Ovestin® (EST), Gino-Canesten® (CLT), Gino Travogen® (ISO), Gyno-Pevaryl® (ECO), Sertopic® (SER) and Dermofix® (SER) as function of time (h). Results are means and bars represent standard deviations (n=3 for each tissue type). * represents

statistically different values between caudal and cranial vagina (two-way ANOVA, $p < 0.05$, Tukey's multiple comparisons test).

Permeation parameters were calculated using the profiles obtained from **Figure IV. 4**, and are detailed on **Table IV. 5**.

The only drug that pointedly accumulated on tissues was EST from Blissel® ($22.7 \pm 3.7\%$ on caudal and $27.1 \pm 3.7\%$ on cranial vagina). This active substance did not surpass the epithelial barrier, however it had affinity to the biological substract, which is expected since estriol is used in vaginal atrophic menopausal symptoms. This connexion with the tissue could be helpful to regenerate the epithelial layer largely injured during menopause^{44,384,385}. On the contrary, EST contained in Ovestin® was not quantifiable in tissues, although approx. 20% of the applied drug permeated the membranes (28.9 ± 2.4 and $21.0 \pm 2.4\%$ in caudal and cranial vagina, respectively). FEN, despite its higher diffusion rate, it did not permeate the membranes and neither largely accumulated in tissues (1.2 ± 0.3 and $0.9 \pm 0.3\%$ on caudal and cranial vagina, respectively). This could be explained by the fact that the biological surrogate is actually a limiting step for fenticonazole.

Only after 8 h of experiment, CLT was quantifiable on the receptor chambers. This drug, along with ECO and SER (coming from Sertopic®) were the only ones to have significant differences between the J_{ss} calculated for the two types of tissues. In all experiments, except Gino Canesten® and Sertopic®, the caudal vagina seemed to be more permeable (despite statistical differences were not always found). When comparing these results with the ones obtained in the release studies it is clear that permeation only occurs (or is detectable) mainly after the 8 h. This further reinforces the idea that vaginal semisolids should be designed to be applied before bed, so they can have a residence time enough to allow drug release, and then the release amount is able cross the biological barriers and exert their therapeutic action, if the desired action is systemic. If it is local, the night administration enhances the formulations contact with the vaginal lining, favouring the local therapy (during day time is more probable to leak). Experiments with vaginal excised tissue to assess vaginal drug permeation for the drugs also included in this study are not reported on the literature. Only a formulation containing clotrimazole loaded into nanostructured lipid carriers for the treatment of fungal vaginal infections was studied, however PBS alone was used as receptor medium and CLT solubility was not considered²⁵⁵. The scarce literature on the subject and the fact that we are dealing with commercial formulations, from which we do not have full information on excipients quantitative composition, could represent a difficulty to discuss and integrate our new findings on the scientific community. Nonetheless, it is truly not our objective to evaluate the products herein tested. These products were chosen, exactly because we are certain that they are largely studied, and having been through clinical trials, and also being marketed, some of them for so long, assures that they are safe, effective and can be used as model products for testing new methodologies, as the ones proposed in this study, and in others already published by our group³³¹. It was not found a significant difference between using caudal or cranial vagina in *ex vivo* vaginal porcine permeation studies, which on one hand could mean

that any of these tissues are representative of the vaginal epithelia, or on the other hand could indicate that both tissues portions should be studied. In fact, as it can be seen on **Figure IV. 4**, although significant differences were not extensive concerning the tissues and formulations included in this study, it was clear that vestibule (caudal) could allow is generally more permeable considering the timeframe used for permeation, and the permeated total drug amounts (**Table IV. 5**). Also, they were shown to be more consistent (lower SDs). Indeed, cranial vagina tissue has more *ruggae* on its constitution and this could explain the higher deviations between samples (tissue irregularities could congregate thicker and thinner parts). So, as a general appointment and taking into consideration the reasons appointed above, we conclude that caudal vagina epithelium should be preferred over the cranial, not only because it has more histological similitudes with the human vaginal epithelium but also because it promotes higher permeation rates (representing a “safer” strategy to study permeation since it will hardly result in false negative outcomes) and more consistent results.

Table IV. 6: Drug recovery from the formulation (%), *ex vivo* permeation and *in vitro* release parameters. P_{app} (apparent permeability coefficient); J_{ss} (steady-state flux); NC (<LLOQ) (Not calculable because concentrations were below the lower limit of quantification); * represents statistically different from the histological tissue in comparison (One-way ANOVA, Tukey's multiple comparisons test, $p < 0.05$). $n=3$ tissues in *ex vivo* permeation experiments and $n=6$ in *in vitro* drug release studies. When drug quantification was <LLOQ, a 0 $\mu\text{g/mL}$ concentration was assumed.

	Recovery from formulation (% of drug)	ex vivo permeation												in vitro release	
		Caudal vagina						Cranial vagina							
		Total permeated amount (% of drug)	Donor Chamber (% of drug)	Tissue (% of drug)	Mass balance (%)	P _{app} (cm/s)	J _{ss} (µg/cm ² min)	Total permeated amount (% of drug)	Donor Chamber (% of drug)	Tissue (% of drug)	Mass balance (%)	P _{app} (cm/s)	J _{ss} (µg/cm ² min)	Diffusion rate (µg/cm ² /h)	r ²
CLT (Gino Canesten®)	95.8	3.2±2.0	88.7±7.9	5.5±8.4	97.5±12.0	2.943x10 ⁻⁷ ±1.808x10 ⁻⁷	1.07x10 ⁻³ ±6.20x10 ⁻⁴	10.0±3.5	79.3±1.1	5.3±0.5	92.2±3.5	9.077x10 ⁻⁷ ±3.208x10 ⁻⁷	3.38x10 ⁻³ ±9.30x10 ⁻⁴ *	25.1	0.9862
ECO (Gyno-Pevaryl®)	98.4	17.6±1.2	77.1±12.7	2.9±0.4	97.5±13.4	1.602x10 ⁻⁶ ±4.621x10 ⁻⁷	6.16x10 ⁻³ ±1.94x10 ⁻³	9.8±1.2	96.1±12.7	0.4±0.4	106.3±13.4	8.931x10 ⁻⁷ ±1.066x10 ⁻⁷	3.16x10 ⁻³ ±7.027x10 ⁻⁵ *	NC (<LLOQ)	
EST (Ovestin®)	104.4	28.9±2.4	70.3±2.5	<1 µg/mL (LLOQ)	99.3±0.1	2.363x10 ⁻⁷ ±5.971x10 ⁻⁷	7.20x10 ⁻⁴ ±2.10x10 ⁻⁴	21.0±2.4	78.3±2.5	<1 µg/mL (LLOQ)	99.3±0.1	1.979x10 ⁻⁶ ±1.900x10 ⁻⁷	6.00x10 ⁻⁴ ±1.452x10 ⁻⁵	20.9	0.9977
EST (Blissel®)	87.2	<1 µg/mL (LLOQ)	60.3±1.5	22.7±3.7	82.9±4.4	NC (<1 µg/mL - LLOQ)		<1 µg/mL (LLOQ)	69.5±1.5	27.1±3.7	96.5±4.4	NC (<1 µg/mL - LLOQ)		13.3	0.9602
FEN (Lomexin®)	118.5	<10 µg/mL (LLOQ)	87.0±3.6	1.2±0.3	88.2±3.9	NC (<10 µg/mL - LLOQ)		<10 µg/mL (LLOQ)	94.0±3.6	0.9±0.3	94.9±3.9	NC (<10 µg/mL - LLOQ)		4805.5	0.9422
ISO (Gino Travogen®)	88.9	14.4±1.7	102.2±0.7	1.5±0.4	118.1±1.0	1.315x10 ⁻⁶ ±1.555x10 ⁻⁷	4.60x10 ⁻³ ±5.30x10 ⁻⁴	12.0±3.3	97.5±8.2	0.6±0.3	110.0±7.7	1.118x10 ⁻⁶ ±3.093x10 ⁻⁷	3.92x10 ⁻³ ±9.60x10 ⁻⁴	55.6	0.9891
SER (Dermofix®)	82.4	22.3±7.5	74.5±14.2	0.4±0.4	97.2±19.5	2.027x10 ⁻⁶ ±9.462x10 ⁻⁷	6.37x10 ⁻³ ±1.47x10 ⁻³	8.8±7.5	96.7±14.2	0.3±0.4	105.9±19.5	7.975x10 ⁻⁷ ±6.871x10 ⁻⁷	2.97x10 ⁻³ ±2.61x10 ⁻³	NC (<5 µg/mL - LLOQ)	
SER (Sertopic®)	104.6	4.6±3.0	90.7±5.0	0.6±0.1	96.0±17.7	4.227x10 ⁻⁷ ±1.232x10 ⁻⁷	1.59x10 ⁻³ ±4.00x10 ⁻⁴	9.5±3.0	98.9±15.0	0.4±0.1	108.7±17.7	8.604x10 ⁻⁷ ±2.723x10 ⁻⁷	3.46x10 ⁻³ ±9.90x10 ⁻⁴ *	NC (<5 µg/mL - LLOQ)	

IV.2.3. Conclusions

In vitro drug release and *ex vivo* drug permeation studies represent tools that shall support researcher to predict drug performance *in vivo* at early stages of product development. In this study, we were able to validate a quantification methodology using HPLC-DAD and applying this to different matrices, not losing quality and resolution in partial validations. Furthermore, we managed to assess the *in vitro* release profiles of eight vaginal semisolids, as well as their permeation profiles, having previously studied the drugs solubility in order to maintain sink conditions. Furthermore, following previous results of our group, we have investigated if there were differences in performing *ex vivo* permeation studies using the porcine vaginal model, when collecting a proximal or a distal tissue within the vaginal tube. No extensive significant differences between these tissues were found, but we concluded that the caudal vaginal could be more suitable for vaginal permeation experiments since it conducts to more reproducible and consistent results. The inclusion of this methodological approach in preclinical stages of products development, could optimize cost-efficiency of new formulations development process by predicting *in vivo* efficacy and safety profiles.

CHAPTER V

GENERAL DISCUSSION

Laboratorial research is, currently, particularly dedicated to discover or optimize former strategies for drug delivery¹⁻³, and the vaginal route of administration has been considered an excellent alternative since it enables both local and systemic delivery⁴⁻⁸. Vaginal drug delivery is accomplished through several dosage forms that are as simple as powders, and as complex as the intravaginal ring, designed for the controlled delivery of hormones¹⁶. Drug delivery efficacy mainly relies on the dosage form ability to promote adequate drug concentration at the targeted action site¹⁷⁻²¹. Several studies have concluded that women prefer and better accept and use, among vaginal formulations, the semisolids, preferably, gels^{22,23}.

In fact, gels might represent the vaginal dosage forms that has been investigated the most in recent years. They can be developed to be stimuli-sensitive systems, which can modify their behaviour once the environmental conditions change¹⁰⁷⁻¹¹⁰. Specifically, aqueous-based gels not only, generally, provide better safety profiles, but also, do represent flexible vehicles that can be improved by means of polymer science and nanotechnology^{51,111,112,115-122}. Since vaginal semisolids are usually committed with discomfort issues the use of strategies like bioadhesion^{93,173} is valuable to avoid interferences with women's daily life, while allowing higher drug bioavailability with smaller absorption variations, and so lower incidence of side effects¹⁷⁻²¹.

Under another perspective of vaginal products development, methodologies improvement to better characterize new or re-newed formulations are needed. As several efforts have been made to conduct investigation to better understand the vaginal epithelial mechanisms^{6,42} here involved, this may benefit characterization methods, that if perfectly aligned with the target epithelia knowledge, can contribute to more successful product development, along with high levels of innovation in the product itself. Indeed, these optimized characterization processes are not only essential in the first steps of development, but are also extremely useful for monitoring batches in quality control or to assess the impact of formulation changes in the industrial context. Thus, it is of great importance to implement accurate and reproducible vaginal semisolids performance evaluation.

Recently, a strategy of patient-driven product development is also currently reflected in policies such as the biopharmaceutics risk assessment roadmap (BioRAM), which intends to optimize drug product development and performance by using therapy-driven target drug delivery profiles as a framework to achieve the desired therapeutic outcome. Simultaneously, clinical relevance is directly correlated to early formulation¹⁴³⁻¹⁴⁵. That means that a full assessment of a final formulation candidate should be performed during its global development, rather, than first selecting the active substance, and then step-by-step increase formulation complexity with intermediate characterization. In this context, jumping to a screening of final complete formulations candidates ahead of the final process development is clearly cost and time-saving.

That was exactly the strategy applied in this thesis work, nevertheless, not for formulations development properly, but in improving the characterization methods. While recent publications on innovative vaginal products are largely devoted to microbicide

formulations, antimicrobials, specifically, antifungals, and oestrogens are no doubt the most prevalent in use due to the clinical relevance of vaginal disturbances, as vaginal infections, and post-menopause atrophy related symptoms. This means that engagement and safety/risk assessments similar to those related to microbicides design should be applied to other vaginal product categories¹⁴⁶⁻¹⁵⁰.

In view of optimizing methods while gathering experimental data from widely used vaginal products, the research work of this thesis started with the selection of vaginal semisolid formulations that were currently commercialized in Portugal (but also representative of the international market, i.e. EU and USA), and therefore, to which easy access and wide interest on the obtained results were assured. Moreover, they should fill three product categories: antimicrobials, oestrogens and products with reported experimental work on technological/safety characterization. The importance of characterizing antimicrobial preparations is obvious due to the prevalence and recurrence rate of vaginal infections. These facts, conduct to difficulties in the clinical setting, once antimicrobial drugs resistance is an inevitable issue¹³². So, in one hand these drug products represent the most often used by reproductive-aged women and on the other hand they actually represent an opportunity for improvement regarding some technological properties, since most of these formulations are actually classical formulations. Oestrogens were added to this evaluation setting, since they represent another important area of vaginal semisolids usage, as local therapies for atrophic symptoms mainly after menopause^{384,386,387}. In addition, two largely described and studied products, Universal Placebo and Replens® were included in this study to serve as reference/control products^{158,321}. They are well-established as safe and/or technologically-advanced vaginal semisolids, being these the reasons why they have been selected to serve in this work as waiving products, in order to guarantee that the employed methods where being optimized in the right direction (the Universal Placebo should present a safe profile that correlates with the findings of clinical trials involving microbicides, while Replens should present high acid-buffering capacity and bioadhesion). To sum up, the products included in the present set of studies were: Gino-Canesten®, Sertopic®, Dermofix®, Gyno-pevaryl®, Lomexin®, Gino Travogen®, Dalacin V®, Ovestin®, Blissel®, Colpotrophine®, Universal Placebo and Replens®.

Vaginal semisolids evaluation itself began with their technological characterization, which included insights from the chemical, the physical and the physiological point of view. This part of the work series consisted on improving traditional characterization methods by using physiological parameters in order to construct predictive tools to characterize close to ideal vaginal semisolid formulation whatever target it may have (as long as it is defined). Products were characterized in terms of: pH and buffering capacity in a vaginal fluid simulant (VFS); osmolality - directly and upon dilution in VFS; textural parameters (firmness, adhesiveness and bioadhesion) using vaginal *ex vivo* porcine epithelium; and viscosity (including VFS dilution at 37°C and after administration on an *ex vivo* model).

The pH values of vaginal products, by itself, does not allow for a full prediction of pH compatibility with the vaginal environment, in view of its safety. It is more relevant to further assess the ability of these formulations to actually change the physiological pH once administered (pH-buffering capacity) by correcting altered pH or maintain it within the assumed healthy range. This parameter, especially when determined in VFS, contributed to a better understanding of what will happen *in vivo* regarding pH changes after the formulation is applied into the vagina¹⁵⁹. For this reason, it is not only important to understand the ability of acidic products to maintain this pH after contacting with successive amounts of an alkaline solution (representing a less acidic vaginal fluid), but also to assess the resistance offered by less acidic products to gain lower pH after mixing with acidic solutions which mimic the vaginal fluid with normal pH.

The results obtained concerning osmolality showed that it might be of special interest to consider its measurement also in physiological dilutions. The proposed test using normal simulant (considering composition and the amount that is believed to be present in the vagina to dilute the applied dose) clearly indicated that, although plain products might have osmolalities above the recommended limits¹⁶⁵ (1200 mOsmol/kg maximum as proposed by the WHO for vaginal lubricants), the resulting osmolality of the dilution in physiological fluids could be compliant with the recommendation. Furthermore, it was clear that the extent of reduction on osmolality for hyperosmolar products was clearly dependent on the product composition since, while for some products only a discreet effect was noted (e.g. Lomexin® osmolality reduced from 1446 mOsm/Kg - direct- to 1210 mOsm/Kg in VFS), for other products osmolality upon dilution in VFS represented less than 30% of the directly measured osmolality (e.g. Colpotrophine® and Ovestin®). So, for future osmolality determinations a complete assessment should not only comprise the direct measurement but also the one made after dilution in the estimated physiologic amounts of VFS. Moreover, it would be important to consider specific disease related characteristics of the vaginal environment that may affect the outcome of this dilution, such as the increased volume or altered (less acidic) pH of vaginal discharge that characterizes bacterial vaginosis and that may affect the performance of antibacterial products. Interestingly, the osmolality of the resulting dilution has the capacity to early predict the *in vivo* formulation behaviour, representing an initial strategy for safety foresee.

The textural parameters studied were firmness and adhesiveness. Their assessment was accomplished by a physic-mechanical method and the objective was to find a correlation between these two parameters, which however, we did not found. Bioadhesion, on its turn, expressed as a mechanical measurement having the vagina as the attachment structure, was found to have a moderate to strong uphill positive linear correlation with adhesiveness determined by pure mechanical methods. This meant that bioadhesion could be predicted by adhesiveness, a test that does not require the use of biological surrogates and can easily and quickly be performed in earlier stages of product development.

Viscosity was, as expected, found to be highly dependent on temperature^{26,287}. However, the variation observed, was nor proportional nor similar among all formulations. Each

formulation showed its own behaviour, once more driven by their specific composition. This could mean that measurements directly made on formulations at room temperature do not represent the viscosity that they acquire after administration, and that those should only be performed for comparative studies. These results sustain that the selection of prototypes intended to proceed in the development process should be based on measurements that mimic the vaginal environment. The test using the *ex vivo* porcine vagina as an organic administration model brought valuable information, with results that were different from those of the dilution at 37°C, supporting its inclusion as a model that better predicts the formulations' rheology after administration.

After the technological profiling of a formulation has been concluded, a preclinical safety assessment is required, in order to complete its characterization, confirming the previous tests that had already been done under a safety perspective. This approach included *in vitro* cellular models, an *ex vivo* tissue model and an *in vitro* organotypic model.

Considering the cellular models, two *in vitro* cytotoxicity tests were performed: MTT and NRU upon uterine (HEC-1A), cervical (HeLa) and vaginal (VK2 E6/E7) cell lines, in compliance with ISO/EN 10993-5 guide for *in vitro* evaluation of vaginal medical devices. The vaginal cell line VK2 E6/E7 showed to be the one that gave the more accurate calculation of TC₅₀ (half-maximal Toxic Concentration) among the three cellular lines on the MTT assay. In fact, this is the most representative cellular model for vaginal products testing, although it was not sensitive to the NRU assay.

Likewise, we have developed and optimized a strategy to determine tissue viability using an *ex vivo* porcine vaginal model by using the MTT reduction assay and histological analysis¹⁸⁵. We showed that it is possible to collect tissues from the porcine model with approximately 15% variability in thickness, and the first optimization step resulted in variation coefficients lower than 25%, when testing negative and positive controls. The inclusion of the proposed *ex vivo* toxicity model in preclinical safety assessment can lead to a better simulation to the *in vivo* conditions. This model, has proven to be reliable and it has low economical expenditures when compared with the 3D reconstructed tissues available on the market. That is a key point why these *ex vivo* tissues could represent valuable tools for scientific research, when early stages of the preclinical development of drugs/products is the focus. Moreover, the *ex vivo* models take into consideration an inter-individual variability that is not present in manufactured reconstructed models, and that in fact, is closer to the variability encountered further on *in vivo* studies.

Safety assessment was further proposed to be performed though an organotypic *in vitro* model adapted from the HET-CAM assay, undergoing full validation for eye irritation testing¹⁹⁸. In this assessment, vaginal semisolid medicines and lubricants currently marketed were tested along with the Universal Placebo formulation which safety has been clinically shown. Nonoxynol-9 (N-9), a known vaginal irritant, was enrolled as positive control (concentrations ranging from 0.001 to 100% (v/v)). The assay was conducted according to the ICCVAM - Recommended test method (NIH Publication No. 10-7553 - 2010). The studied vaginal

formulations showed low potential for irritation. N-9 was classified as a severe irritant at concentrations above 2%, which corroborates clinical data from the literature, envisaging a possible *in vitro/in vivo* correlation. IS (B) was considered as having a better classification output. Although still requiring further validation, the HET-CAM assay showed to be sensitive enough to differentiate vaginal products according to irritancy potential.

Finally, *in vitro* drug release and *ex vivo* permeation studies were performed under efficacy and safety perspectives. To accomplish this task, firstly a validation of an analytical method (HPLC-DAD) was performed to quantify six different molecules present in the vaginal semisolid products. Secondly, *in vitro* drug release studies were performed to characterize drug diffusion through the formulation, to evaluate if it was a limiting step for permeation. *Ex vivo* drug permeation studies were performed using two histologically distinct tissues collected from different regions of the porcine vagina (caudal and cranial portions). Permeability comparisons between these two tissues were assessed. The validation of the HPLC-DAD method was successfully accomplished according to FDA, EMA and ICH requirements. Receptor media for both experiments were selected based on solubility studies to assure *sink* conditions. Permeation through the vaginal tissue was found to be dependent on the type of formulation tested and not directly defined by the release rate, although it was a consequence of it. Caudal vagina was slightly more permeable than the cranial portion although statistical differences were not evident for all studied products. Results deviations were more pronounced with the cranial vagina due to the presence of extensive *ruggae* in it. Therefore, although *ex vivo* drug permeation through different regions of the porcine vagina did not vary extensively, the use of caudal vagina for these studies may yield more reproducible results.

All over the experimental work not only the characterization methods were optimized but also important experimental data were gathered from commercial products widely available and considered as safe. These data will be essential to allow for comparisons with new products under development on the basis of “non-inferiority” considering each of these characteristics.

The results obtained with this thesis are particularly valuable for the company where the research work took part and that supported it; however, once the papers herein presented are published, they would be applicable worldwide, being expected that these results will be largely accepted by the scientific community. The application and performance of these methodological approaches will certainly benefit several workgroups, not only the ones working on vaginal semisolids products, but also other vaginal dosage forms. Furthermore, the optimized procedures conducted concerning the *ex vivo* porcine model, were demonstrated as interesting and with high potential to be submitted as a proposal, for alternative methods to laboratory animals, to the respective regulatory authorities.

Summarizing, the objectives defined at the beginning of this thesis were fully accomplished. Now, a complete preclinical performance profiling of vaginal semisolid products, based on a set of methods ranging from simple technological characterization to complex studies like drug *ex vivo* permeation, is defined and available. All the methods here described,

alone or combined, do certainly represent valuable tools to be applied in new products development and characterization both in industrial and academical environments.

CHAPTER VI

CONTRIBUTIONS FOR ENHANCING THE COMPETITIVENESS OF THE HOSTING COMPANY (LABFIT-HPRD, LDA)

Labfit is an R&D company dedicated to the development of innovative pharmaceuticals classifiable as medicines, medical devices or cosmetics, as well as, the provision of R&D services in the health area, thus working with a high level of technology, not only in fundamental research, but also on applied and translational research. Furthermore, part of the work developed by Labfit is specialized in topical application products with a special focus in the area of gynaecology.

Therefore, the objectives of this thesis were completely aligned with the company's strategy and contributed for Labfit's expertise on topical products characterization. Indeed, all the research hither presented contributed for enhancing the competitiveness of Labfit as these results were transferred from the academia to the company as:

- A complete technological characterization service. A full assessment of vaginal semisolids galenic and technological characterization method was systematized. From simple assays, such as organoleptic characteristics, pH and osmolality determination, to others more complex, like pH buffering capacity, texture and viscosity. Moreover, a physiological approach has been applied to these methods, not only because they were discussed taking into account the healthy vaginal environment and the *in vivo* behaviour, but also, because actually some assays were concomitantly performed using a vaginal fluid simulant and an *ex vivo* porcine model;
- An integrative *in vitro* and *ex vivo* safety characterization service. This was optimized considering the current normatives and consists on alternative method to experimental animals that includes two different cellular and tissue toxicity method and an organotypic method for vaginal irritation testing which broadens the scope of application of the methods previously performed at Labfit;
- An HPLC validated method for quantification of the most representative active substances present in vaginal semisolid formulations. The method was validated according to FDA, EMA and ICH requirements and has great potential to be applied in Labfit as services for product characterization in this diversity of molecules;
- A drug permeation test that combines both *in vitro* and *ex vivo* models allowing a full access to vaginal drug delivery either intended to be local or systemic;
- The combination of the results obtained with this work contributed to start the construction of an automatic in-line database. This will be used by Labfit during product development or optimization and during interpretation of results from products received as samples for characterization, helping to evaluate their technological properties and safety.

The scientific knowledge together with the laboratory skills obtained with this work were translated into written procedures and, further qualification of researchers for these methods being converted in new or more accurate services provided by Labfit, as well as, an international recognition of this company as an expert on topical pharmaceutical products for vaginal application.

CHAPTER VII

CONCLUSIONS AND FUTURE REMARKS

Optimized preclinical performance assessment of vaginal semisolid products seems to represent a key point in new products development. Improvements in methodologies turn them time saving, cost-efficient and very helpful in the decision-making process. The assumption that physiological parameters (composition and amount of fluids, body temperature and even movements) should be considered, can further expedite this process while bringing assessment close to real life conditions.

The work developed during this thesis supports the concept that a primary technological characterization under a physiological perspective is essential. It was highlighted that pH buffering capacity, osmolality and viscosity determined using the proposed methodological adaptations were considered focal points to be addressed during products' development. The importance of giving attention to vaginal antimicrobial and oestrogenic formulations at the same level of that payed to microbicides, was stressed. Another point evidenced by this investigation was that polymer-based strategies could be useful in re-formulating products in order to overcome problems of leakage and discomfort, and improve efficacy. Since polymeric formulations demonstrated more favourable characteristics regarding the vaginal epithelia.

Concerning preclinical safety assessment, three strategies have been combined in this thesis. First, *in vitro* cellular models were used to assess toxicity using conventional and standardized methods. Second, an *ex vivo* tissue model was established and adequately optimized to improve the complexity level and robustness of toxicity assessment over the first method. Third, an organ-based *in vitro* method, already in validation by regulatory agencies for testing irritation in other biological surrogates, was effectively transposed to vaginal irritation testing. These set of methods can not only be applied to the pharmaceutical industry and research, but also to the cosmetic, hygiene, and medical devices industries. Moreover, the *ex vivo* model herein proposed, can further be applied to the development of primary cell cultures, as a surrogate for permeation and metabolic studies, and even to evolve to more complex systems, including co-cultures with microorganisms.

The multi-drug HPLC-DAD quantification method developed and validated in view of this work has demonstrated a great potential of applications, including the study of different types of vaginal products with distinct active substances but also, as it is able to quantify six different molecules in just one run, it would be valuable for quantification of drugs in new developing products that contain combinations of these active substances. Moreover, it could be applied not only on drug release and permeation, but also could be useful for drug quantification in formulations under testing, for example, in stability studies or quality control after production.

Product performance on the developed *in vitro* drug release and *ex vivo* drug permeation studies, demonstrated a great potential to predict drug performance *in vivo* at early stages of product development. This combined methodology conducted to a full assessment of drug release and permeation profiles in eight different vaginal semisolids. Once again, in drug permeation, the porcine *ex vivo* model has confirmed to be an efficient, manageable and applicable tool in several testing methods. Furthermore, a precise study conducted over

anatomical and histological featuring's of this *ex vivo* model, have determined that it is essential to assign which type of tissue is collect from the porcine vagina.

Following this methodological definition and optimization that have allowed for the objective characterization of a representative group of currently marketed semisolids, this tool may be used to assess the individual influences and contributions of those excipients frequently used in vaginal formulations for the overall performance of the product, regarding each of the parameters under study, by using a single, well studied base (such as the Universal Placebo). The database that has been prepared in the current project will then be enlarged with data from excipients and will become a powerful tool to assist the rational design or optimization of vaginal products towards fulfilling the vaginal semisolids market considering clinical needs and women's preferences.

To sum up, the objectives proposed in the beginning of this thesis were accomplished, and now, a complete assessment on preclinical performance of vaginal semisolid products has been described and applied to formulations that are currently marketed worldwide. This assessment being based on technological and safety evaluations that assume physiologic parameters is worth to be applied in new products development and characterization, either on academia or on an industrial environment.

The here established set of assessment methods will from now on be adopted by Labfit in research as well in current testing services.

CHAPTER VIII

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CHAPTER IX

APPENDICES

APPENDIX A

Review article that contributed to CHAPTERS I and IV in the published format:

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Studies and methodologies on vaginal drug permeation[☆]

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ABSTRACT

The vagina stands as an important alternative to the oral route for those systemic drugs that are poorly absorbed orally or are rapidly metabolized by the liver. Drug permeation through the vaginal tissue can be estimated by using *in vitro*, *ex vivo* and *in vivo* models. The latter ones, although more realistic, assume ethical and biological limitations due to animal handling. Therefore, *in vitro* and *ex vivo* models have been developed to predict drug absorption through the vagina while allowing for simultaneous toxicity and pathogenesis studies.

This review focuses on available methodologies to study vaginal drug permeation discussing their advantages and drawbacks. The technical complexity, costs and the ethical issues of an available model, along with its accuracy and reproducibility will determine if it is valid and applicable. Therefore every model shall be evaluated, validated and standardized in order to allow for extrapolations and results presumption, and so improving vaginal drug research and stressing its benefits.

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1. Introduction

Researchers are now devoted to find new forms or to re-discover safer and more effective alternative routes for the administration of drugs that

are poorly absorbed orally or precociously suffer metabolism [1–3]. The vaginal route has been considered of great interest for drug delivery, since it enables both local and systemic drug delivery [4,5], allowing for the absorption of peptide and other macromolecules, and even nanoparticles [6–8].

The vaginal route provides different advantages over the oral one but it is not deprived of inconveniences [9,10]. Its large surface area, rich blood supply, ability to bypass hepatic first-passage, avoidance of gastrointestinal side effects, and relatively high permeability to a wide range of molecular weight drugs are some of its physiological characteristics that

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contribute to its pharmacokinetic advantages [11–14]. However, drug absorption through the vagina may be affected by variations in epithelial thickness and by changes in the vaginal milieu composition that occur as a consequence of age dependent and cyclic physiological conditions or sexual intercourse. Moreover, leakage and self-cleaning action of the vaginal tract may reduce drug bioavailability [5,15]. Furthermore, general disadvantages of vaginal drug delivery include its obvious gender specificity, cultural background limitations and personal hygienic care interference [12,14].

Vaginal drug delivery systems include solutions, semisolids (creams, ointments and gels) and solid formulations (tampons, capsules, pessaries, suppositories, films, sponges, powders and special controlled release devices like the intravaginal ring) as well as other types of formulations such as aerosols and particulate systems integrated in adequate drug delivery systems [16]. Efficacy of drug delivery systems will rely on their ability to promote adequate drug concentrations at the targeted site of action. When a systemic effect is the objective through this route, drugs must be transported across the epithelium to gain access to dermal vessels and the systemic circulation [17]. On the other hand, when a local effect is the goal, as is the case for some antimicrobials and microbicides, retention of the drug at the surface of the vagina is desirable with low grade of absorption [18–21].

Drug permeation studies are mandatory for vaginal drug administration when systemic delivery is intended, and are important for safety characterization when the objective is to limit the activity to the vaginal wall surface or its contents. The *in vitro* models developed to predict drug permeation not only provide information on absorption rates and efficacy, but also help in investigating and understanding the pathogenesis of various microbiological diseases [22–24].

So, applicable and reproducible test assays are important to provide comprehensiveness about mechanisms of permeation, absorption and mode of action of active substances for vaginal application [25] and also to characterize vaginal drug delivery systems and the formulation ability to either promote or avoid permeation through the vagina [26, 27].

Previously published scientific studies on this subject are quite unclear concerning the specific meaning of permeability and permeation terms. We assume that, by definition, *permeability* is the property of membranes or barriers of an organ or structure of being permeable to substances, while *permeation* denotes the ability of substances, like drugs, to permeate through a membrane/barrier [28]. Nevertheless, it is clear that this difference is not assumed uniformly and that the terms “drug permeation” and “drug permeability” have been and still are used interchangeably in the literature.

2. Vagina: anatomy, histology and physiology

The vagina is described as an expandable, longitudinally S-shaped, fibromuscular, collapsed canal showing at transverse cross-section an H configuration, with the anterior and posterior walls contacting each other in current conditions. It extends from the cervix of the uterus to the vestibule [2,29,30], presenting approximately 7–10 cm in length, more than 4 cm in width and 150–200 µm in thickness. The posterior wall is longer than the anterior one, a consequence of the asymmetrical position of the cervix at the vaginal vault [25,31–33].

The vagina is the female sexual organ by definition, and though it normally does not harbor glands, it is usually referred as a mucosa. In fact, despite not having a secreting role, the vaginal epithelial surface is actually coated by a thin layer of fluid that includes endometrial, cervical and vestibular secretions, residues of urine and products of cellular autolysis, and variable amounts of vaginal wall transudate [34,35]. Additionally, the composition of the vaginal fluid varies according to age, menstrual cycle and health status condition [31,36]. For instance, the vaginal pH is acidic (3.5–4.5) in healthy women during the reproductive age but it fluctuates along the different stages of the menstrual cycle and it is also dependent on coitus frequency, the amount of cervical mucus

present in the vagina, the amount of vaginal transudate and it also varies along the vagina being higher close to the cervix and lower at the anterior fornix [2,35]. The maintenance of the pH is accomplished by lactic acid bacteria, mainly *Lactobacillus* spp., since these microorganisms metabolize into lactic acid the mono and di-saccharides that result from the autolytic breakage of desquamated vaginal cells glycogen [31].

The vaginal wall consists of various cell layers: nonkeratinized stratified squamous epithelium, lamina propria, muscular layer and tunica adventitia (covering only their proximal segments). The lamina propria is constituted of connective tissue rich in blood and lymphatic vessels draining to the internal iliac vein, this explaining why the absorbed products do avoid the hepatic circulation as an initial passage [30]. The vaginal cell turnover is estimated to replace 10–15 layers in a week [37]. The nonkeratinized stratified squamous epithelium, settled on glycogen containing keratinocytes but also integrating other cell types (such as macrophages and Langerhans' cells), is grounded on the lamina propria consisting of fibroblasts, elastic and collagen fibers, vessels and nerves, and defense cells, mainly polymorphonuclear leukocytes and occasional lymph nodes [38]. The vaginal epithelial cells are disposed according to different stages of differentiation, identifiable through different keratins expression, such as K10 and K13, being the differential expression arrangement function of the cell location within the epithelium (Fig. 1) [39]. Numerous folds and microridges called “rugae” are present in the epithelium, largely increasing the vagina's surface area and providing distensibility [7].

The vaginal innervation depends on two types of sources: a peripheral one providing a highly sensible lower quarter segment and an autonomic fiber network in the upper vagina, which is more sensitive to stretch than to touch or to painful stimuli. This explains why women do not feel discomfort when using continuous intravaginal drug delivery systems [7].

Several conditions influence vaginal physiology: hormonal balance, pregnancy, pH, microflora and age, being the last one the best biomarker for epithelium layer thickness, enzyme concentrations and vaginal fluid production [41]. These vaginal characteristic changes influence drug permeation as it depends mainly on the superficial layer characteristics, as thickness, cell tightness, and lipids composition and organization in the intercellular space [25,42,43].

3. Drug absorption from the vagina

Drug dissolution in the vaginal fluid and epithelial penetration are the two key steps for a drug to be absorbed through the vagina. As a result all factors associated with vaginal physiology and formulation profile will greatly influence the success of drug delivery to the target [6,7]. Even though the vagina is not a real mucosa, drug transport is accomplished in a multi-way mechanism similar to the other biological membranes. Drug absorption can occur passively or actively. Passive mechanisms include the transcellular route, through the cells' membrane and the paracellular route, representing a diffusion process through intercellular fluid and tight junctions [44,45]. Tight junctions and other intracellular junctions (adherens junctions and desmosomes) are present in the vaginal and cervical epithelium having a higher expression in the endocervix. Although the uppermost layers of the ectocervical and vaginal epithelium are devoided of tight junctions, these and other intercellular junctions have been identified right beneath the most apical epithelium layers. The study of these junctions is particularly important to understand, for example, the invasion of microbes and drug permeation. Tight junctions are composed of transmembrane proteins (occludin, claudins and junctional adhesion molecules – JAMs) which contact across the intercellular space and create a seal to restrict paracellular diffusion of molecules across the epithelial sheet. Furthermore, tight junctions have a structural role in epithelial polarization by limiting the mobility of membrane-bound molecules between the apical and basolateral domains of the plasma membrane of each epithelial cell. In general, tight junctions are

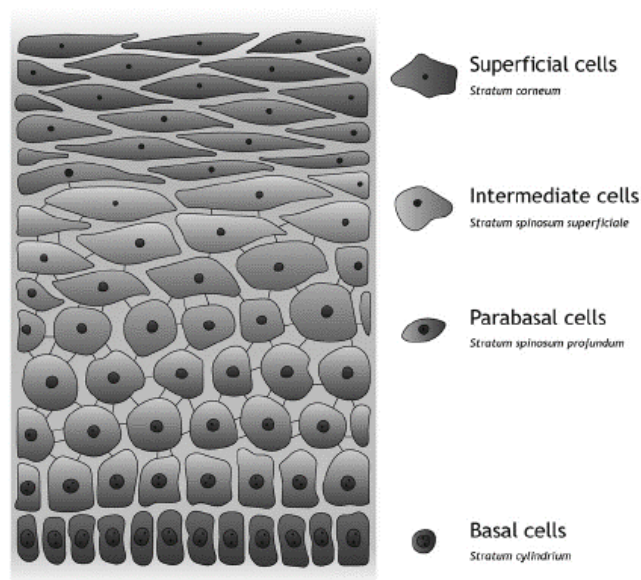


Fig. 1. Schematic representation of non-keratinized stratified squamous vaginal tissue. Adapted from: Schnell, *Citología y Microbiología de la vagina* [40].

responsible for sealing the epithelial barrier as well as the selective passage of small ions and fluid [44].

The active transporters generate gradients across the barriers, being the mechanism either a primary transcellular process or a secondary one. While the latter is indirectly coupled to adenosine triphosphate (ATP) energy, the primary active transport directly utilizes ATP during the transport cycle [46]. Nevertheless, previous studies on vaginal permeation of drugs show that most of the active substances permeate the vagina through diffusion mechanisms [17]. Lipophilic substances, such as steroids, are mainly absorbed by the transcellular route [31], in contrast to hydrophilic drugs which follow the paracellular diffusion mechanism [17]. Low molecular weight lipophilic drugs are more likely to be better absorbed than larger molecules or even hydrophilic drugs, independently of their molecular weight [5].

Several factors affect drug absorption from the vagina. Both vaginal fluid amount and composition interfere in drug dissolution before transport. While an excessive fluid content promotes a “washing” effect that decreases drug retention, cervical mucus presence is able to increase bioadhesion [31]. Estrogens, due to epithelial cell proliferation stimulation, influence drug pharmacokinetics through the vagina, as a consequence of increased blood irrigation, transudation and cervical mucus production. Consequently, when a systemic action of a drug is desirable, several factors shall be taken into account, like the epithelium layer thickness, the enzymatic and hormonal cyclic changes, the vaginal pH (that interferes with ionization of electrolytic drugs) and sexual stimulation and intercourse [47,48].

Additionally, vaginal administration of a substance, being either a drug or an excipient, may cause irritation, inflammation and damage, leading to barrier disruption and so enhancing not only its absorption but also the penetration of infectious agents [25,49]. Drugs and excipients' physicochemical characteristics are also relevant for determining their absorption [50,51]. The excipients present in the formulation have also an important role both in drug release and absorption profiles [7]. Molecules' lipophilicity, ionization, molecular weight, surface

charge and chemical nature represent the most relevant properties that determine their ability to permeate both the apical and basolateral membranes of the epithelial cells [24,52].

4. Vaginal drug permeation methodologies

The ethical and practical constraints to the use of humans and their organs to assess drugs and formulations' efficacy and safety, led researchers to create and develop alternative methods and models to predict drug permeation through the vagina. These include cells, tissues (both organs and culture systems) and full animals [25,53–56]. Technical complexity, economic expenditures and ethical issues determine if a model will either be valid, applicable and reproducible or if it is not trustworthy to be used in laboratory research. Table 1 gathers some of the main advantages and disadvantages of models to be applied for predicting vaginal drug permeation.

4.1. *In vitro* models

Several *in vitro* studies have been conducted to characterize the vaginal barrier concerning drug permeation [57–60]. These models comprise cell-based and reconstructed tissue models.

Over the last decades, cell-based *in vitro* models for drug permeation prediction allowed the use of simple, economic, reproducible and ethically accepted methodologies in the earlier stages of drug development. These models use both immortalized cells and primary cell cultures that are able to proliferate in monolayers, simulating the intended epithelium, both structurally and functionally [24]. Cell culture models can be easily manipulated, in terms of culture conditions and parameters, and represent good drug absorption predictors. Human cell lines or primary cell cultures circumvent problems related to the use of animal tissue on *in vitro* models [61].

The main limitation of *in vitro* models, result from the impossibility of integrating cell external factors (diseases, age, hepatic and renal

Table 1

Advantages and disadvantages of models applied for predicting vaginal drug permeation. Adapted from Costin et al. (2011) [38].

	<i>In vitro</i>	<i>Ex vivo</i>	<i>In vivo</i>
	Cell culture models Reconstructed tissue	Tissue explants	Laboratory animals
Advantages	Cultures are relatively inexpensive and easy to grow.	Full cell structure (epithelial, connective, immune). Better tolerance to formulations. Parallel efficacy testing.	Complete organism features addressed: structurally, functionally and physiologically.
Disadvantages	No barrier function for cell cultures and no systemic component. Organotypic cultures tend to be more permeable than <i>in vivo</i> and have no vascular component.	Limited number. Institutional review board approval required. More technically demanding. Variability.	Time and cost expensive. Data still difficult to correlate to humans. Regulatory and ethical issues.

functions, environmental characteristics) that are imperative for the organism function. Although, extrapolation of *in vitro* results to humans must be done very carefully [24], the *in vitro*–*in vivo* correlation represents an important tool for prediction of *in vivo* pharmacokinetics. Clark et al. in 2011 tried to compare results obtained *in vitro* with *in vivo* for a predelinical evaluation of a microbicide in the form of a micronized and non-micronized drug. However, *in vivo* pharmacokinetic results (in rabbits) failed to corroborate the higher permeability for the micronized drug obtained with reconstructed tissue *in vitro* studies [24].

Drug physicochemical and biopharmaceutical properties as well as physiological and environmental organism characteristics should be considered. Among them are drug solubility, acid dissociation constant (pKa), drug permeation, octanol–water partition coefficient (logP) and the environment fluid pH [62]. Therefore, *in vitro* permeability studies also play a key role in determining a formulation strategy in order to assist solubility, dissolution and stability [63].

In the 90's Gorodeski et al. developed a method based on the harvesting of cervical–vaginal cells collected from women, aged from 22 to 49 years, undergoing hysterectomy. Initially, a primary cell culture of human ectocervical epithelial cells was obtained (hECE) [64], that included histologically normal cells only. This technique was applied to permeation mechanistic studies. To this purpose, the cells were grown on collagen-coated ceramic-based filters and differentiated into a 5–12 cell layers to form a squamous stratified epithelium, mimicking the biological characteristic human cervical–vaginal epithelium [65, 66]. When these cells were compared to a cervical cell line (CaSki), the latter ones showed to be simpler to maintain and a better alternative

to the primary cultures obtained from hysterectomy specimens. In this study, it was also evidenced the epithelial nature of CaSki cells and their improved differentiation when grown on filter support [67]. Although they are easier to grow, maintain and test, cell line cultures correspond to immortalized cells and consequently may not mimic real cell behavior. From this point of view, primary cell cultures should be valued since they represent a more reliable biologic model.

When different cellular models were developed and compared, hECE, the primary culture, was settled as a model for ectocervical epithelium, while ECE16-1 and CaSki, both immortalised cell lines, expressed phenotypic characteristics of squamous metaplastic cervical epithelium and endocervical epithelium respectively [68]. Later on, CaSki endocervical cell line was shown to be able to form cell monolayers and even bi/trilayers, depending on cell seeding density, and was proven to be useful for transport mechanisms studies [69–71]. These experiments resulted in interesting findings about the importance of tight junctions and their influence on transepithelial electrical resistance (TEER) [66] and the increase of TEER by seminal fluid [70]. They also contributed to improve the knowledge about the modulation of vaginal permeability to pyranine (used as a model drug for paracellular transport) by factors such as estrogen stimulation and aging (due to changes in the resistance of both the lateral intercellular space and the intercellular tight junctions) [72–76], or extracellular ATP and Ca²⁺ (due to their effects at the tight junctions level) [67,75]. All this research resulted in substantial basic knowledge for vaginal permeability studies. Table 2 resumes the *in vitro* models developed for the study of drug permeation through the vaginal barrier.

Table 2*In vitro* models developed for predicting drug permeation across the vaginal barrier.

	<i>In vitro</i> model	Source	Permeation features studied	References
Cell-based	HEC-1A – cell line	Human endometrial carcinoma.	Drug permeation and solubility. Excipients influence on drug permeation and solubility.	[23]
	hECE – human ectocervical epithelial cells – primary culture CaSki – human cervical epithelium cells – cell line C-33A – cervix cancer cell line cells Primary vaginal epithelial cell culture	Human biopsy, histologically normal cells. Human epidermoid carcinoma. Human cervical retinoblastoma.	Transepithelial electric resistance. Transepithelial electrical conductance. Permeability to pyranine. Transepithelial electrical conductance.	[64–66,68–71,75,84]
Reconstructed tissue	Human reconstructed vaginal tissue integrating normal or SiHa cell line and Langerhans Cells (LCs)	Human biopsy, healthy woman. Cell line from a squamous cell cervical carcinoma.	Drug permeation. Study of the transepithelial route followed by pathogens.	[85,87] [78]
	VEC – vaginal–ectocervical tissue model (EpiVaginal®)	Normal human-derived vaginal ectocervical epithelial and dendritic cells – MatTek.	Safety evaluation of compounds applied on vaginal epithelium. Transepithelial electrical resistance. Sodium fluorescein leakage. Inflammatory cytokine release.	[86–89]
			Potential use for screening and assessment of the irritation, penetration metabolism, or efficacy of active ingredients or final formulations for vaginal application.	[56,79–83]
	HVB® – Human vaginal epithelium	A431 cells derived from a vulval epidermoid carcinoma grown on a polycarbonate filter – SkinEthic.	Screening and assessment of the irritation, penetration, metabolism, or efficacy of active ingredients or final formulations for vaginal application.	[89–91]

Permeability studies using either primary cultures or cell lines of vaginal/cervical epithelial cells have been described in the literature and are herein discussed [72,74,76–78]. Cell lines represent more standardized models than primary cultures, since they are ready to use without rising important inter laboratory variability results and interpretations. Comparative studies on permeability differences between primary cell cultures and cell lines represent an important topic to be explored, for example using the TEER measurement. The simplicity of these *in vitro* models can lead to limitations in terms of *in vitro*–*in vivo* data correlation when compared to methods based on human vaginal and cervical tissue explants. The importance of establishing these correlations justifies the need to proceed with tests that better mimetize *in vivo* mechanisms by using reconstructed tissue, tissue models (*ex vivo*) and animals (*in vivo*).

Regarding tests using reconstructed tissue, the EpiVaginal® model (MatTek Corporation, Ashland, United States of America), is a commercial vaginal–ectocervical (VEC) tissue-like model widely used for microbicides testing. It consists of a 3D-culture of non-transformed human vaginal–ectocervical epithelial cells grown on polycarbonate cell culture tissue inserts. The VEC tissue model has proven to be highly reproducible and represents a non-animal method to assess the irritation of contraceptives, microbicides, and vaginal-care products [57,79]. Despite being largely used for studying prevention of pathogen

transmission [80,81] and tissue toxicity [79], the complexity of the multi-layer structure makes it a promising option for permeability studies. Several other authors have already used EpiVaginal® as model to predict vaginal permeability to chemicals [82,83] (Table 2).

4.2. *Ex vivo* models

Ex vivo models have been used for predicting drug permeation through the vaginal barrier by using either human or animal vaginal tissue. *Ex vivo* tissues allow not only for drug permeation assays but also enables histological analysis to assess differences before and after drug application/permeation [92]. While some authors may classify these *ex vivo* experiments as *in vitro* experiments, the two concepts are considered to be different for the purpose of this review. Laboratorial reconstructed tissues are herein classified as *in vitro* assays, as they are grown in artificial milieu, while *ex vivo* assays require tissue excision from animals or humans, and their use in conditions that preserve their original biological and physical characteristics for a certain period.

Fresh human cervical explants are clinically obtained from women undergoing planned hysterectomies. In the USA some repositories provide fresh samples for research institutes and industries, nevertheless it is doubtful that the samples available are sufficient to extensive experiments, such as the toxicological ones [38]. Researchers are concerned

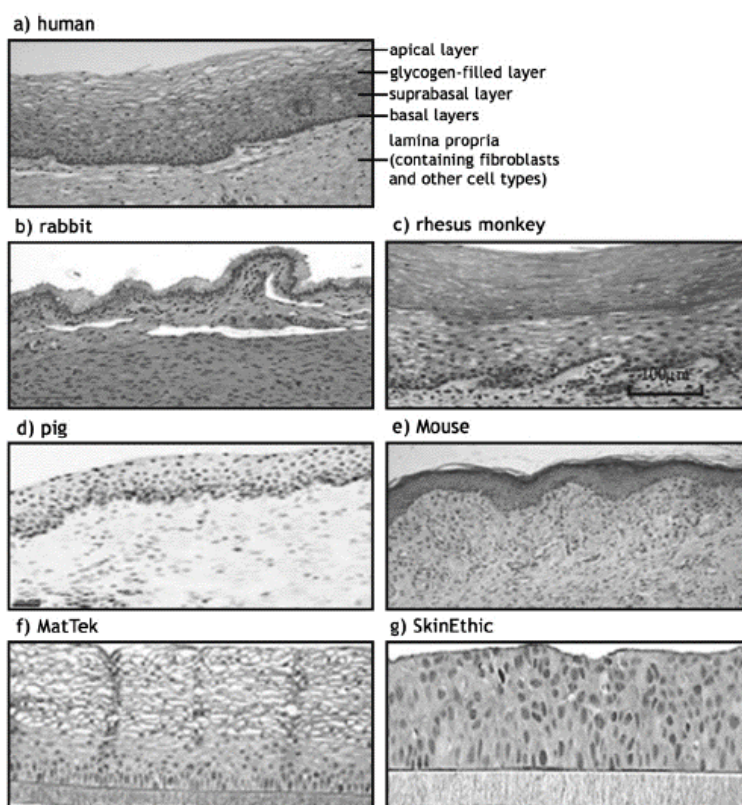


Fig. 2. Comparison between H&E-stained vaginal epithelium of: human (a), rabbit (b), rhesus monkey (c), pig (d), mouse (e), EpiVaginal from MatTek Corporation (Ashland, MA, USA) (f) and HVE – Human vaginal Epithelium from SkinEthic (Nice, France) (g). Reproduced from Reference [38] with kind permission from FRAME.

Table 3
Permeability methods for predicting drug permeation across the vaginal barrier.

Membrane	Membrane source and preparation	Drug/substance	Permeation experiment characteristics	Quantification method	References
Franz cell systems					
EpVaginal® VEC-100-FT	Commercially available.	UC781, atenolol and antipyrine	Receptor solution: 0.5 mL of the assay medium on the basolateral side of the insert. Sample collection: 200 µL were taken from the receptor compartment at different times, and the same volume of the fresh assay medium was replaced back to the same compartment. Receptor solution: 11 mL of phosphate-buffered saline, at 37 °C. Franz cells: area of $1.0 \pm 0.1 \text{ cm}^2$. Sample collection: 100 µL at fixed time intervals up to 8 h. Sample preparation: filtration. TEER was measured to check monolayer formation and integrity. Receptor solution: HBSS with poloxamer 407, pH 7.3.	LC	[83]
Porcine vaginal tissue	Tissues collected from female laboratory pigs. The tissue was frozen in SVF at -20°C immediately after sacrifice. Samples were cut in 1 cm square pieces and defrosted before experiments at ambient temperature in the presence of SVF. Seeding and culture was performed on transwell permeable supports for 8 days.	Clotrimazole		HPLC	[108]
Cadsi cell monolayers		Polymetric nanoparticles of dapivirine		HPLC	[116]
Porcine vaginal tissue	Obtained from a slaughter house; transported in RPMI-1640 medium, and used within 2 h from sacrifice. Thickness of $800 \pm 300 \mu\text{m}$.		Receptor solution: 8 mL of HBSS containing 0.2% (w/v) of poloxamer 407 under magnetic stirring (300 rpm). Franz cells: exposure surface was 1.77 cm^2 . Sample collection: samples were periodically collected from the receptor chamber and the volume replaced with fresh HBSS. Cell monolayers were also collected either at 1 or 4 h after being washed with PBS, lysed and analyzed for soluble dapivirine. Receptor solution: 7.5 mL oxygenated Ringer buffer at pH = 7.4, at 32 °C. Sample collection: every 30 min up to 4 h. Sample preparation: aliquots and extracted tissues (cryotomized at -25°C to 20 µm slices) were prepared for liquid chromatography. Receptor solution: 4.5 mL of phosphate-buffered saline (PBS), pH = 7.4, at 37 °C. Franz cells: 7 mm of diameter; 0.385 cm^2 of area. Sample collection: 200 µL were removed from the receiver compartment at various time intervals for a total period of 2 h. Sample preparation: Liquid scintillation cocktail, 2.5 mL.	Fluorescence microscopy HPLC	
Bovine vaginal tissue	Obtained from a local slaughterhouse and transported in isotonic NaCl solution. Unstripped vaginal tissue was cut into 5 cm ² segments.	Ketoconazole, propranolol, furosemide, atenolol, ranitidine, curcumin		LC	[103]
Cervical and vaginal human tissue	Tissues collected from women undergoing hysterectomy for benign conditions.	Tritiated water		Liquid scintillation	[17]

(continued on next page)

Table 3 (continued)

Membrane	Membrane source and preparation	Drug/substance	Permeation experiment characteristics	Quantification method	References
Human vaginal tissue	Excised vaginal epithelial tissue from routine reparative surgical procedures. Tissue was flash frozen immediately in a liquid nitrogen atmosphere and stored at -70°C . Before experiments as thawed slowly at 4°C and cut into cylinders, approximately 6 mm in diameter and 4 mm deep, using a dermatological punch. Obtained from a daughter house. Tissues were thawed in PBS (pH 7.4) for 10 min. The thawed specimens were cut into 4 mm diameter sections.	5-aminolevulinic acid	Receptor solution: 10 mL of PBS pH 7.4 Diffusion cells: modified Franz diffusion cell and exposed to a water-soluble, ALA-loaded, sample preparation: the tissue was cryostatally sectioned and the stratal concentration of radiolabeled ALA determined using scintillation spectroscopy. Receptor solution: PBS pH = 7.4, at 20°C Diffusion cells: flow-through diffusion cells (exposed areas 0.039 cm^2) Sample collection: PBS at 20°C was pumped through the acceptor chambers at a rate of 1.5 mL/h and collected, by means of a fraction collector, at 2 h intervals for 24 h (100 μm). Sample preparation: to each sample collected, 15 mL scintillation cocktail was added.	Scintillation spectroscopy	[106]
Using Chambers	Porcine vaginal tissue	17β -estradiol, ranitidine, vasopressin, oxytocin and water	Receptor solution: PBS pH = 7.4, at 20°C Diffusion cells: flow-through diffusion cells (exposed areas 0.039 cm^2) Sample collection: PBS at 20°C was pumped through the acceptor chambers at a rate of 1.5 mL/h and collected, by means of a fraction collector, at 2 h intervals for 24 h (100 μm). Sample preparation: to each sample collected, 15 mL scintillation cocktail was added.	Liquid scintillation	[104]
	Millipore Durapore® 0.45- μm pore size	miniCD4 M48U1	Receptor solution: 13 mL of SFV without BSA, at 37°C Diffusion cells: area of 2 cm^2 Sample collection: 1 mL at fixed time intervals up to 24 h.	Surface plasmon resonance	[117]
Vaginal macaque tissue	Collected from naïve female Cynomolgus macaques (<i>Macaca fascicularis</i>) and rinsed with Ringer solution (pH 6.8).	M48U1	Receptor solution: Ringer solution Sample collection: until 2 h, 0.1 mL was removed		
Flow-through diffusion cells	Porcine vaginal tissue	Tritiated water	Receptor solution: PBS 0.01 M, pH 7.4 at 37°C , pumped through the receptor chamber at a rate of 1.8 mL/h Diffusion cells: 5 mm diameter orifice, with an exposed epithelial surface of 0.20 cm^2 Sample collection: the perfusate was collected in scintillation vials at 60 minute intervals for up to 19 h using a fraction collector Sample preparation: the samples were mixed with a scintillation cocktail.	Liquid scintillation	[118]
Human vaginal tissue	Obtained from excess tissue removed from postmenopausal patients after vaginal hysterectomies. Only normal specimens were used.	Tritium radioisotopes of water, 17β -estradiol, arecoline, arecaidine, Sumatriptan, cyclosporine, benzalkonium chloride, benzolaliprene, vasopressin, 4.4-kd and 12-kd fluorescein-isothiocyanate-labeled dextrans.	Receptor solution: PBS pH = 7.4, at 20°C Diffusion cells: area of 0.039 cm^2 , flow-through chambers. Sample collection: PBS at 20°C was pumped through the acceptor chambers at a rate of 1.5 mL/h and collected, by means of a fraction collector, at 2-h intervals for 24 h (100 μm). Sample preparation: to each sample collected, 15 mL scintillation cocktail was added.	Liquid scintillation	[76,77,94–97, 119–126]

with the difficulty to obtain and maintain human explant specimens at the laboratory. To overcome these difficulties, freezing of specimens has been considered and no negative influences in permeability parameters were found for this tissue conservation method. In fact, no statistical differences were found between fresh and frozen tissue permeability results when tissues were collected 1 h after the surgical excision, transported in PBS or culture media at 5 °C, frozen with methanol/dry ice or liquid nitrogen, and kept frozen at –80 °C [17]. Previous studies conducted by van der Bijl et al. showed that freezing at –85 °C had no effect on tritiated water permeation through vaginal tissue, after tissues transport in a transport fluid, and transferred to laboratory within 1 h, snap-frozen in liquid nitrogen and stored at –85 °C for up to 6 months [93–97]. Although, permeability studies may not be influenced by tissue freezing–thawing, the impact on vaginal irritation is not clear, since cellular viability might be affected [38,98]. Meanwhile, the difficulties encountered with the human tissue samples impelled the demand for other alternatives, such as animal tissues.

Vaginal tissue obtained from different animal species such as rodents, rabbits, monkeys, cows and sheep have been used for permeability studies [99–103]. Vaginal absorption may be significantly different when comparing the animal and the human models.

Bovine vaginal tissue was used to test drugs permeation, as surrogate for human tissue due to anatomical and physiological similarities [103]. Nonetheless, bovine epithelium exhibits significant histological differences when compared with human vaginal epithelium and is not widely applied. In contrast, porcine tissue represents the animal model most commonly applied for this purpose. Porcine *ex vivo* tissue specimens are convenient, since they are simple to handle, inexpensive to obtain and easy to work comparing to the whole animal. Human and porcine vaginal tissues present substantial histological similarities [25, 98] (stratified squamous epithelium supported by connective tissue – Fig. 2). Additionally, porcine vagina can be easily accessed through local slaughterhouses.

Although, generally, porcine vaginal tissue seems a good *ex vivo* permeability model for human vaginal tissue extrapolation it is of great importance to validate a permeability study concerning other tissues or *in vitro* tests. It has already been shown that for hydrophilic molecules, (water and vasopressin, for example), the porcine vaginal tissue is an accurate *in vitro* permeability model of human tissue, however for more lipophilic molecules (such as oxytocin) the flux through porcine vaginal tissue was 53% higher than the corresponding estimated value through the human vaginal tissue [104]. *Ex vivo* tissues are mainly tested for drug permeation using one of three established techniques: Franz cell systems, flow through diffusion cell and Ussing chambers.

4.3. Permeation tests

The United States Pharmacopeia (USP) clearly defines the performance tests for topical drug products, including vaginal administration products, which are focused on the assessment of *in vitro* drug release. It is defined that this test must be performed in vertical diffusion cell (VDC) systems, which by their simplicity can provide reliable and reproducible measurements of drug release from semisolid formulations. Franz cells represent the most often used VDC, on the top of which 200–400 mg of the testing formulation is placed, over the membrane to be studied. The application site can be variable in size, but is typically of 15 mm of diameter. For vaginal drug delivery systems the assay temperature must be kept at 37 ± 1 °C. Samples of the recipient fluid are collected up to 4–5 h, and the volume withdrawn is replaced with fresh medium. Sink conditions must be assured, meaning that the receptor medium must have a high capacity to dissolve the drug, and the receptor media should not exceed 10% of the concentration of the standard at the end of the test. This test should be done with two runs of 6 cells in order to document the release rate [105].

Although USP recommendations point towards the use of synthetic, inert and highly permeable membranes in the context of these drug

release studies, the interest in performing *in vitro* permeability studies using biological membranes is evident. Biological tissues/membranes are more similar to the *in vivo* conditions and may provide information on bioavailability of the drug. However, they are also more complex, and data obtained can be more difficult to interpret and discuss. Generally, the difficulties to be encountered are related to tissue preparation and system setting-up. The membrane preparation depends on tissue provenience. Since human tissues are difficult to obtain, frozen and thawed samples are frequently used since previous studies demonstrated that this procedure does not affect tissue permeability [96,106].

Permeation systems intended to study drug release from vaginal dosage forms include Franz cell systems, flow through diffusion cells and Ussing chambers, further described in Sections 4.3.1, 4.3.2 and 4.3.3, respectively.

Perfusion cells are commercially available in different configurations and sizes concerning tissue exposure areas, donor and receptor chambers. In terms of exposed tissue, the surface areas are quite variable ranging from 0.039 to 3.14 cm² in published studies (see Table 3). Also the receptor chamber volumes are diverse. Every detail must be addressed in experiments preparation, since they should not only follow pharmacopoeial recommendations, but also, integrate physicochemical characteristics of the substances under study. In addition to maintaining a physiological temperature throughout the study, the selection receptor fluids should be adequate since it is crucial to preserve tissue integrity over time, in order to mimic physiological conditions. The temperature should also be adequate and identical to *in vivo* conditions.

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organization which harmonizes policies in developed countries and has issued several publications concerning the safety assessment of chemicals and chemical preparations (www.oecd.org). Since, for example, vaginal lubricants can be classified, for marketing purposes, as medical devices or even hygiene products (i.e. they are not medicines, and so do not necessarily follow the pharmacopoeia specifications), manufactures should follow the same methodology on characterizing products to standardize procedures. Patterning the manufacture and testing methods provide better awareness of the product, even on already commercialized products [107]. The OECD series on Testing and Assessment Number 28, clearly describe for skin permeability testing, the types of studies (*in vivo* or *in vitro*) and the detailed procedure data. These include the species to be selected together with the number, sex, housing and feeding conditions of animals used in *in vivo* studies; and for *in vitro* studies the diffusion cells type, the receptors fluids characteristics and skin preparation. Additional considerations regarding the testing substance are also described. No specific guidelines are available for vaginal (or genital) products. Since the vaginal route plays an important role both on local and systemic delivery, the availability of validated vaginal models and regulatory recommendations similar to those for skin permeability studies is of extreme relevance. Also, the European Union Reference Laboratory for alternatives to animal testing (EURL-ECVAM) has not yet validated *in vitro* methods for vaginal route studies, as it has been done for skin irritation and corrosion (OECD test guidelines 431 and 439). Nevertheless, since there are commercially available *in vitro* reconstructed tissues mimicking the vaginal epithelium (such as EpiVaginal® and HVE®), they might need, in a future, an international recognition (meaning validation), so they can be widely applied with reinforced confidence on the results obtained.

4.3.1. Franz cells

Several *in vitro* studies have already been conducted to characterize the vaginal barrier in terms of drug permeation using a Franz cell system [57,58]. Additionally this model constitutes the methodology of reference in the USP for topical drug products. The system is composed of two compartments: a donor compartment (upper chamber) and a receiver compartment (lower chamber) (Fig. 3). A membrane is placed

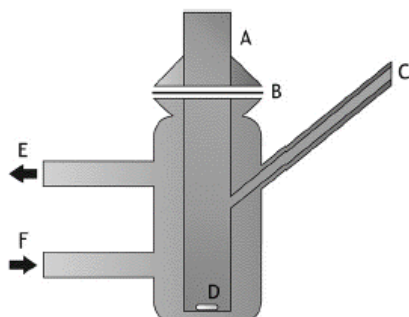


Fig. 3. Schematic representation of a Franz cell. (A) donor compartment; (B) membrane; (C) sampling port; (D) stirbar; (E) water outlet; (F) water inlet.

between the two compartments. The formulation to be tested is placed on the top of the membrane, in the donor compartment which does not need to be filled with a liquid. Heated water circulation is maintained around the receptor chamber all over the process, usually at 37 °C to mimic human body temperature. The stirbar guarantees the receptor solution homogeneity. Samples can be accessed over time by collecting aliquots through the sampling port.

A phosphate-buffered saline (PBS) solution, with a physiological pH is usually applied in the receptor chamber [17,108] despite oxygenated Ringer buffer has also been used [103]. Tissue thickness and area must be taken into consideration in results calculations. USP recommends the use of synthetic and inert membranes in which the product to test is placed, letting the system to saturate for 30 min (equilibration period). The USP indicates that the epithelial side of tissues must face the donor solution. Aliquots from the receptor compartment are collected at predefined time intervals. The aliquots volume withdrawn must be replaced with the same volume of receptor solution to ensure sink conditions. Afterwards, aliquots should be analyzed through diverse analytical techniques for identification of drug in the collected sample. Membrane/tissue should also be checked for histological modifications after the permeability study.

Steady-state flux (J_{ss}) and apparent permeability coefficient (P_{app}) can be calculated based on the Fick's First Law of Diffusion, with the following equations – Eqs. (1) and (2).

$$J_{ss} = \frac{Q}{A \cdot t} \quad (1)$$

$$P_{app} = \left[\frac{dQ}{dt \cdot A \cdot C_d} \right] \times 60 \quad (2)$$

being Q the quantity of substance crossing membrane [counts per minute (cpm)], A the area of the membrane exposed (cm^2), and t the time of exposure (min); the units for J_{ss} are $\text{cpm cm}^{-2} \text{ min}^{-1}$. In the second

equation, dQ/dt is the slope of the curve of Q versus t , A remains the surface area and C_d is the initial concentration of the compound in the donor compartment (cpm/cm^3). Therefore, the units for P_{app} are cm/s [93].

The permeability chamber design may be responsible for results variability. Classic Franz cell system used in permeation studies can lead to stasis and/or accumulation of perfusate in the receptor chamber, which can be overcome by using a diffusion flow-through system [58]. Concerning the experimental conditions, temperature stands as an essential parameter to be controlled since drug diffusion depends on temperature. Also, tissue origin and the experiment duration must be optimized to get the best profit of permeability studies [25]. Additionally, researchers should be aware of intrinsic biological variability which is obviously more pronounced in tissues and reconstructed tissues, than in synthetic membranes.

Several investigation studies have already been performed using Franz cell systems to perform *in vitro* drug release studies on vaginal products (Table 3).

4.3.2. Flow through diffusion cells

Flow-through diffusion cells have been developed to overcome an identified limitation of static diffusion chambers: the possible decrease of diffusion gradient following the accumulation of the permeating drug in the receptor compartment. These diffusion cells present the same principle as those previously described since they are based on the product application at one side of the tissue/membrane (donor), with permeation monitored, through aliquot collection and analysis, on the receptor side. However, these systems are characterized by the use of a perfusion fluid, below the membrane surface, to collect the permeating substance and allow for a concentration gradient across the tissue that better resembles the *in vivo* conditions. Also, the sampling can be further facilitated by collecting the effluent using a fraction collector (Fig. 4). The flow-through diffusion cell allows for diverse benefits: automatic sampling; easy maintenance of sink conditions; mimicking the physiological assessment of percutaneous absorption; simulation of the blood flow subjacent to biological membranes [109].

However, the receptor volume is critical since, to completely wash the permeating substance, the pumped volume must be many times the volume of the receptor. This requires the receptor volume to be small so that the volume of effluent from the cell is manageable [110]. A different type of modified flow diffusion cells was proposed by Bonferoni et al., characterized by a flow stream on the donor chamber in order to measure the washability of semi-solid formulations [111].

4.3.3. Ussing chambers

Ussing chambers are also used for the experimental measure of drug permeation through biological barriers. These systems allow not only for temperature control, but also for oxygen circulation in the two chambers and measurements of electric parameters to monitor tissue viability [112].

The Ussing chamber system, introduced by Hans Ussing in the early 1950s [113], is composed of a chamber, a water jacket to maintain physiological temperature, a gas stream system to maintain a physiological

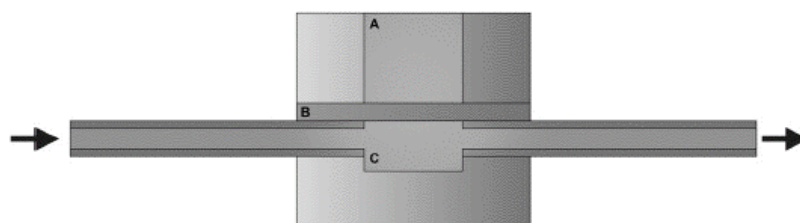


Fig. 4. Schematic representation of a flow through diffusion chamber. (A) donor compartment; (B) membrane; (C) receptor compartment.

buffer while providing a gas lift circulation and, if needed, an amplifier and/or a data acquisition system (Fig. 5). Although they were initially developed to study ion transport mechanisms across the epithelia, Ussing chambers have also been applied to drug permeation studies (Table 3). Epithelia are polar structures possessing an apical (also referred to as mucosal) and basolateral (or serosal) side. The electrolytes, non-electrolytes, and H₂O movement across the membrane once quantifiable can be extremely useful in substance permeation studies. Ussing chambers not only support native human or animal tissues but also membranes derived from cell monolayers and reconstructed tissues. Specifically, cells can be grown on monolayers in a culture insert and then placed on a Snapwell chamber, which is provided by the apparatus manufacturer and can be used in the Ussing chambers system. In the Snapwell system the drug to study is dissolved in the donor fluid. For formulation studies several difficulties may arise as is the case of semi-solids testing since the viscosity of the product or the solution obtained after dissolution may impair gaseous and nutrient exchange therefore negatively impacting cell viability and the obtained results. Semi-solid formulations can be directly applied on tissues or for instance on reconstructed tissues while for cellular permeation studies, a previous dilution is necessary to avoid the occlusive effect.

The barrier to permeate is placed between the two chamber halves (Fig. 5). Diverse technical variations of these systems have been developed to meet the appropriateness for different experiments including vertical and horizontal Ussing chambers. The central purposes of Ussing chambers technique are electrophysiological and diffusion-based studies, or moreover a combination of both [114].

The Ussing chamber system consists of two functional parts: the chamber itself and the electrical circuit. The electronic circuit measures the current (*I*) and voltage (*V*) which allow for the calculation of resistance (*R*), and also more complex parameters like impedance and capacitance [115]. Depending on the type of fluid circulation Ussing

systems can be classified into two types: the circulating chamber and the continuously perfused chamber. The circulating chamber is U-shaped, embraces the experimental solution and can be heated and gassed (air, CO₂, O₂ or N₂), in order to oxygenate the liquid contents and guarantee complete convection of the liquid. The U-shape ensures equal hydrostatic pressure on both chamber halves. This chamber is considered to be robust and simple to use. Continuously perfused chambers are not commercially available, but can be manufactured by design with specific configurations. The main purpose of these chambers is to minimize the hydrostatic pressure, avoiding damage to the testing tissue. The flow of the perfusion solutions is accomplished by using peristaltic pumps. The immerse solutions to be applied in the chamber are automatically delivered from reservoirs mounted above the chamber. The flow rate of injection can be regulated. The majority of the chambers are made of Teflon® or Lucite® and are available at various sizes and shapes [114].

Epithelium is distinct from other tissues due to its polarity and tightness. This characteristic derives from the asymmetric distribution of proteins to either the apical or the basolateral side of the cellular membrane. The tight junctions seal adjacent cells, and its formation and permeability determines the resistance (*R*) and, consequently tissue integrity. The resistance is calculated through the following equation – Eq. (3).

$$R = \frac{\rho l}{A} \quad (3)$$

where ρ is the specific resistance module of the material, *l* the length or thickness of the material (which should be constant for each tissue preparation), and *A* the surface area. Considering a specific tissue, *R* can be seen as a sum of resistors, being *R_a* the resistance of the apical membrane and *R_b* the resistance of the basolateral membrane, shunted by a parallel resistor *R_{shunt}*. Therefore, the total transepithelial resistance *R_t* is defined by the Kirchhoff's law expressed in Eq. (4).

$$R_t = \frac{(R_a + R_b)R_{shunt}}{R_a + R_b + R_{shunt}} \quad (4)$$

R_t reflects directly the tissue integrity and can be easily calculated using the Ohm's law – Eq. (5).

$$R_t = \frac{\Delta V}{\Delta I} \quad (5)$$

The easier way to assess *R_t* is to apply a voltage and measure the resulting change in current (called "voltage clamping"). In addition, the ion transport across epithelial tissues generates a transepithelial voltage, *V_{te}*. Another electric parameter to take into account is short circuit current (*I_{sc}*). This current is defined as the charge flow per time when the tissue is short-circuited and is measured when *V_{te}* is clamped to 0 mV. The amount of current required is adjusted and registered. The values different to 0 mV enable a estimation of *R_{te}*, a value that can be applied in the following equation to calculate *I_{sc}* – Eq. (6).

$$I_{sc} = \frac{V_{te}}{R_{te}} \quad (6)$$

In summary, an experiment using Ussing chambers shall consist on three steps. A preparation phase must be performed to ensure chambers, solutions, electrodes validity and readiness. At this time the chambers must be mounted without tissue for watertight verification of the system, while temperature and gassing can also be tested. As a second step it is recommended to perform a control experiment to check for electrical interferences (noise) and offset voltages. This also allows for resistance estimation of the empty chambers. Only after all these verifications, the tissue experiment should be accomplished (third step). The tissue is mounted between the half chambers, allowed for an

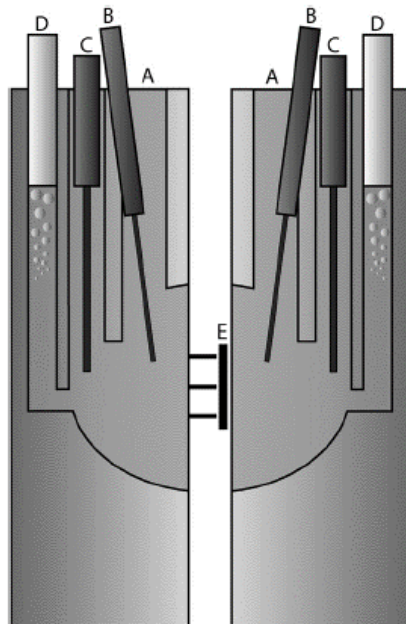


Fig. 5. Schematic representation of a circulating Ussing chamber vertical system. Navicite™. (A) sampling port; (B) current electrodes; (C) voltage electrodes; (D) gasification tube; (E) membrane.

equilibration period, and after a baseline is reached the experiment can begin [115].

Table 3 summarizes the permeability studies (either based on Franz Cell systems, Ussing Chambers and flow through diffusion cells) undertaken for vaginal tissue by several investigation groups.

4.4. *In vivo* models

In vivo models represent the most complete approach to achieve experimental data. However, they are associated with ethical issues, require high cost expenditures and are time consuming. Few animals have been studied for *in vivo* vaginal formulation testing, specifically for vaginal irritation [127–129]. The rabbit vaginal irritation (RVI) model is mostly used since it is the only approved model for the regulatory acceptance of new products [38].

Vaginal permeability testing is scarce on *in vivo* studies, although it could be of great interest especially for systemic drug delivery systems, provided that it is previously validated, ethic, safe and with benefit to new drug and formulation development. Acartürk et al. used normal and ovariectomized rabbits to mimetize different reproductive physiological status, particularly post-menopausal human vagina. Explants of vaginal tissue from these animals were further used for comparative *in vivo/ex vivo* vaginal permeability and enzymatic activity studies [130]. This model was also applied in the study of nonoxynol-9 as spermicide [131].

More recently, the pig model has also been used for *in vivo* experiments. Female large white pigs were used for the determination of zidovudine and polystyrene sulfonate in plasma and vaginal tissue, after the application of an intravaginal bioadhesive polymeric device for up to 28 days. At the end of this period, vaginal tissue was collected for histological analysis, and for substances extraction. Both zidovudine and polystyrene sulfonate were found in low concentrations in plasma, indicating the high retention in the vagina. Additionally no histopathological toxicity was evidenced for this intravaginal bioadhesive polymeric device [132].

5. Conclusions

The vagina is a promising route for drug delivery intending both local and systemic drug delivery. Over the last decades, efforts have been made to conduct investigation to a level of high comprehension of vaginal drug absorption mechanisms. The understanding and application of several techniques to predict drug permeation through the vaginal barrier contributes to the successful selection of drugs and appropriate formulations in the early stages of development. Thus, it is of great importance to implement accurate and reproducible methods to predict drug permeation. The *in vitro* and *ex vivo* tests should be privileged, once an optimal *in vitro*–*in vivo* correlation has been established. Furthermore, tissue explants (*ex vivo*), being more representative in structure and presenting more viability, shall be preferred over *in vitro* cellular methodologies. Standardization and validation of methodologies to be used by different research groups will be obviously valuable for uniform interpretation and extrapolation of test results. Moreover, these tests may be particularly important for the development and characterization of new products intended to reach the market since they provide vital information for marketing and regulatory authorization purposes.

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APPENDIX B

Research article that contributed to the first experimental part of CHAPTER III in the format that has been submitted.

Research article

Optimization and application of *in vitro* and *ex vivo* models for vaginal semisolids safety evaluation

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Abstract

Vaginal semisolids are widely used both for clinical and intimate hygiene goals. Preclinical safety assessment includes cytotoxicity assays upon cell lines to predict potential epithelial irritation, corrosion and sensitization. Furthermore, recent work on tissue explants have highlighted its potential for application on *ex vivo* models. In the present study, we studied traditional and renewed methods for toxicity assessment of vaginal semisolids using pharmaceutical products which are used in clinical practice as antimicrobials (Gino-Canesten®, Sertopic®, Dermofix®, Gyno-pevaryl®, Lomexin®, Gino Travogen®, Dalacin V®) and containing oestrogens (Ovestin®, Blissel®, Colpotrophine®), and reference formulations (Replens®, Universal Placebo). To achieve our outcome, two *in vitro* cytotoxicity tests were performed: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and neutral red uptake (NRU) into uterine (HEC-1A), cervical (HeLa) and vaginal (VK2 E6/E7) cell lines, in compliance with ISO/EN 10993-5 guide for *in vitro* evaluation of vaginal medical devices. Likewise, we have developed and optimized a strategy to determine tissue viability using an *ex vivo* porcine vaginal model by using the MTT reduction assay and histological analysis. The vaginal cell line VK2 E6/E7 conducted to the more accurate calculation of TC₅₀ (half-maximal Toxic Concentration) among the three cellular lines on the MTT assay. In fact, this is the most representative cellular model for vaginal products testing, although it is not sensitive to the NRU assay. It was possible to collect tissues from the porcine model with approx. 15% variability in thickness, and a first optimization step conducted to variation coefficients lower than 25% when testing negative and positive controls. The inclusion of the *ex vivo* toxicity model in preclinical safety assessment can lead to a better approximation to the *in vivo* situation. Furthermore, the application of these models can improve cost-efficiency in early steps of product development.

Keywords: vaginal drug delivery; cytotoxicity; *in vitro* model; *ex vivo* vaginal model

Introduction

The vagina is a promising route of administration when local and/or systemic drug delivery is intended (Alexander et al., 2004; Hussain and Ahsan, 2005; Srikrishna and Cardozo, 2013; Woolfson et al., 2000). The vaginal route comprises diverse pharmacokinetic advantages due to its large surface area, rich blood supply, avoidance of the hepatic first-pass effect and relative high permeability to many drugs (Vermani and Garg, 2000). Nevertheless, several drawbacks, including leakage, inconsistent drug absorption, influence on sexual intercourse and local irritation, need to be addressed during the early stages of product development (Hussain and Ahsan, 2005; Nappi et al., 2006). Ideally, vaginal drug delivery systems should not interfere with either vaginal physiology or women's daily life, while allowing obtaining high drug bioavailability with little variability and low incidence of side effects (Anderson et al., 2013; Chatterton et al., 2004; Dukhin and Labib, 2013; Ensign et al., 2014; Sassi et al., 2004).

Vaginal drug delivery systems include liquid, semisolid and solid formulations (Allen et al., 2011). Semisolids (creams, ointments, gels) have been reported as the most preferred dosage form for self-use by women concerning HIV prophylaxis (Hardy et al., 1998a, 1998b, 1998c, n.d.; Woodson and Holt, 2015). Concerning all these particularities added to women's preference and acceptability patterns for vaginal semisolid formulations (Palmeira-de-Oliveira et al., 2014a, 2014b), a safety- and suitability-driven study is expected to conduct to a better characterization of the already marketed products (which are considered safe based on clinical trials and current use) in view of the development of new products. Furthermore, the improvement of traditional characterization methods by considering physiological parameters that are going to be present after vaginal administration, can provide predictive tools to better characterize new vaginal semisolid formulations and optimize their acceptability and even enhance cost-efficiency at early stages of product development (Machado, 2017).

Over the last decades, efforts have been made to conduct investigation to a level of high comprehension of vaginal epithelium mechanisms (Wu and Robinson, 1996). The understanding and application of several techniques to predict drug toxicity through the vaginal epithelium contributes to the successful selection of drugs and appropriate formulations in the early stages of development (Costin et al., 2011). Thus, it is of great importance to implement accurate and reproducible methods to predict drug toxicity. The *in vitro* and *ex vivo* approaches should be privileged, and preferably an optimal *in vitro/in vivo* correlation should be established (Ardolino et al., 2016). The strategy of patient-driven product development is also reflected in recent policies such as the biopharmaceutics risk assessment roadmap (BioRAM), which aims to optimize drug product development and performance right from the early stages (Dickinson et al., 2016; Selen et al., 2014; Selen and Ph, 2011).

In the field of vaginal administration, research and innovation has been largely focused on microbicide formulations. However, the same safety/risk assessments should be transposed to other vaginal product categories. In fact, preclinical development, i.e. nonclinical access to

toxicological and pharmacokinetics data could be optimized in order to reduce general financial costs and overall time of product development before commercialization (Cox Gad; Fernández-Romero et al., 2014; Lackman-Smith et al., 2010; Lard-Whiteford et al.; Morrow et al., 2014).

Despite being an internal cavity, the vaginal epithelium is constantly exposed to potential pathogenic microbes, microflora, chemicals, and hormonal changes. As minor injuries can occur either mechanically or chemically after products usage/administration, further damage or infection may be promoted (Cunha et al., 2014). Vaginal pharmaceutical, cosmetic and personal care products can occasionally induce undesirable local or systemic side-effects. Still, the standard method for assessing vaginal mucosal irritation is the *in vivo* rabbit vaginal irritation test (Costin et al., 2011; “Guidance for development of vaginal contraceptive drugs”) (ISO/EN10993-5, 2009). Nonetheless, the current mind-set in toxicology is to use alternative *in vitro* methods that reduce, or, even better, replace the use of animals (3Rs), with a refined profile that modulate and predict human, not animal, responses (Costin et al., 2011; Liebsch et al., 2011). This approach is of particular importance in the field of personal care and cosmetic industries since they have to comply with the European legislation, such as the 7th Amendment to the EU Cosmetics Directive that does not allow the marketing of cosmetic products if they, or their ingredients, have been tested for irritation responses in animals (“7th Amendement to the EU Cosmetics Directive” 2013). Safety concerns are currently spotted right through the early stages of development, especially in what concerns to microbicides formulations. This preoccupation was even more highlighted after the unexpected findings of nonoxynol-9 (N-9) in clinical trials (Van Damme et al., 2002), stressing the need for more appropriate *in vitro* assays to predict *in vivo* safety issues.

The cosmetic industry, under the supervision of regulatory agencies (FDA, ECVAM, OECD), has already been evaluating and adopting alternative *in vitro* methods for eye irritation, phototoxicity, and skin irritation, sensitisation, and corrosion (“European Union Reference Laboratory for alternatives to animal testing (EURL-ECVAM),” 2016). However, up to now the cosmetic and pharma industry are still not focused on validating standardised tissue models or cellular assays for vaginal irritation assessment, since this application still represents only a small part of business.

Cytotoxicity assays constitute a gold standard of *in vitro* preclinical evaluations of chemicals in cultured cells. The MTT reduction assay is one of the most common applied cytotoxicity assays. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a water soluble yellow tetrazolium salt, which is converted to an insoluble purple compound (formazan) due to cleavage, within the mitochondria, of the tetrazolium ring by, mainly succinate, dehydrogenase. Formazan does not permeate cell membranes, and consequently it accumulates in metabolic viable cells. The MTT assay was tested for its validity in various cell lines (Mosmann, 1983) and has already been applied to uterine (Youssef Gali et al., 2010c), cervical (Dobaria et al., 2009) and vaginal (Fichorova et al., 1997a) derived cells. Further modifications of the initial protocol described by Mosmann (Mosmann, 1983) were proposed

(Denizot and Lang, 1986; Hansen et al., 1989) in order to improve the repeatability and the sensitivity of the assay.

The Neutral Red Uptake (NRU) assay is also a quantitative colorimetric method. It is based on the ability of viable cells to incorporate and bind (uptake) the red dye within the lysosomes (Borenfreund et al., 1990; Fotakis and Timbrell, 2006; Repetto et al., 2008). Although they are used extensively as convenient and rapid measurements of cell viability, all these methods should be carried out with some caution. In fact, an increase in NR uptake can be induced by lysosomal swelling agents such as weak alkaline substances and osmotic swelling agents. Regarding the MTT assay, some reducing agents and respiratory chain inhibitors could affect the MTT formazan formation. The MTT assay was also found to be significantly influenced by a number of parameters such as medium pH, D-glucose concentration in culture media, and cellular concentration of pyridine nucleotides (Chiba et al., 1998). Additionally, the NRU assay present some advantages over the MTT assay. The former procedure is more sensitive and readily quantifiable. It is at least two times cheaper, presents less interferences, and does not use an unstable tetrazolium salt (Repetto et al., 2008). As a general remark, these assays have, in common with other cell culture procedures, limitations concerning the substance chemical characteristics, say to be volatile, unstable or explosive in water, only very partially soluble, colourant and chemical variably incompatible with the test substance (Freshney, 2000; Repetto et al., 2008).

In the present study, these two cytotoxicity tests, MTT and NRU assay were applied to cervical, uterine and vaginal cell lines in compliance to ISO/EN 10993-5 guide for *in vitro* evaluation of medical devices (ISO/EN10993-5, 2009) (which recommends these tests upon BALB/3T3 clone A31 and NCTC clone 929 cell lines, which are convenient from mouse muscular fibroblasts, not representing a human surrogate) to infer cytotoxicity of final formulations dilution upon *in vitro* models. Furthermore, we have developed a strategy to determine tissue viability upon an *ex vivo* porcine vaginal model using the MTT reduction assay and histological analysis.

Materials and Methods

Tested Formulations

Ten different semisolid vaginal products that are commercially available in Europe and the USA were included in this study: Gino-Canesten® (Bayer, Portugal), Sertopic® (Ferrer, Portugal), Dermofix® (Azevedos Laboratories, Portugal), Gyno-pevaryl® (Johnson & Johnson, Portugal), Lomexin® (Jaba Recordati, Portugal), Gino Travogen® (Bayer, Portugal), Dalacin V® (Pfizer Laboratories, Portugal), Ovestin® (Aspen Pharma, Portugal), Blissel® (ITF Medivida, Portugal), Colpotrophine® (Teva Pharma, Portugal). Replens® (Laboratoires Majorelle, France) (Acartürk, 2009; Adriaens and Remon, 2008; Caramella et al., 2015; Schwartz et al., 2007; Tien et al., 2005; Valenta, 2005) and Universal Placebo (Bygdeman and Swahn, 1996; Clark et al., 2011; Garg et al., 2010; Nachtigall, 1994) were used as reference products, since their toxicity profile is largely described on the literature. Universal Placebo was prepared according to Tien et al. (Tien et al., 2005) by dissolving of 2.7g of Hydroxyethyl-cellulose (2000cP) in 96.3g of water containing 0.85g of sodium chloride and 0.1g of sorbic acid. The final pH was adjusted to 4.4 by adding sodium hydroxide, and the gel was stored at 2-8°C. General characteristics of the studied products are listed in **Table 1**. All products, except placebo and Replens®, are classified as medicines, whilst comprising an active pharmaceutical ingredient (API). Nevertheless, some do not require a medical prescription to be dispensed at pharmacies, which is the case of Gino-Canesten® and Gyno-Pevaryl®.

Materials

Reagents used include Roswell Park Memorial Institute-1640 RPMI (RPMI-1640, Sigma, Germany), Dulbecco's Modified Eagle Medium F12 (DMEM F12, Gibco, USA), Keratinocyte-serum free medium (Gibco, USA), human recombinant epithelial growth factor (hrEGF, Gibco, USA), bovine pituitary extract (BPE, Gibco, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Amresco, USA), Foetal Bovine Serum (FBS, Merck, Germany), Penicillin and Streptomycin (SP, Sigma, Germany), Phosphate Buffer Solution (PBS, Sigma, Germany), Dimethyl Sulfoxide (DMSO, Fisher Chemical, United Kingdom), Triton X100 (Fisher Chemical, United Kingdom), Sodium Dodecyl Sulphate (SDS, Acros Organics, Belgium), Nonoxynol-9/Tergitol (N9, Sigma, Germany), Neutral Red (NR, Acros Organics, Belgium), Ethanol (Manuel Vieira e C^a, Portugal), Glacial Acetic Acid (ChemLab, Belgium), Hydroxyethylcellulose (HEC; Natrosol 250 HX, Ashland Inc., USA), Sorbic Acid (Sigma-Aldrich, Germany) and Sodium Chloride (Merck, Germany). All used chemicals and reagents were of analytic grade or equivalent.

Table 1: General characteristics of the formulations included in the study (information provided by the manufacturer).

	Product Dosage form	API	Excipients
Antimicrobials	Gino-Canesten® vaginal cream	Clotrimazol 10mg/g	Benzyl alcohol, Cetyl palmitate, Cetostearyl alcohol, Purified water, Polysorbate 60, Sorbitan monostearate, Octyldodecanol
	Sertopic® vaginal cream	Sertaconazole 2g/100g	Ethyleneglycol and polyethyleneglycol, Stearate palmitate, Saturated glycerides polyglycolized, Glycerol isostearate, Liquid paraffin, Methylparaben, Sorbic acid
	Dermofix® vaginal cream	Sertaconazole 20mg/g	Tefose 63, Labrafil, Peceol, Liquid paraffin, Nipagin, Sorbic acid
	Gyno-pevaryl® vaginal cream	Econazole 10mg/g	Stearate pegoxol 7, Liquid paraffin, Oleic macrogolglycerides, Butylhydroxyanisole (E320), Benzoic acid, Purified water
	Lomexin® vaginal cream	Fenticonazole 20mg/g	Propylene glycol, Hydrogenated lanolina, Sweet almond oil, Polyglycol esters of fatty acids, Cetyl alcohol, Glyceryl monostearate, Sodium EDTA, Purified water
	Gino Travogen® vaginal cream	Isoconazole 10mg/g	Polysorbate 60, Sorbitan stearate, Cetostearyl alcohol, Thick paraffin, White Vaseline, Purified water
	Dalacin V® vaginal cream	Clindamycin 20mg/g	Propylene glycol, Cetostearyl alcohol, Liquid paraffin, Sorbitan stearate, Benzyl alcohol, Cetyl palmitate, Stearic acid, Polysorbate 60, Purified water
Oestrogens	Ovestin® vaginal cream	Estriol 1mg/g	Octyldodecanol, Glycerol, Cetyl alcohol Stearyl alcohol, Polysorbate 60, Sorbitan stearate, Lactic acid, Chlorhexidine hydrochloride, Sodium hydroxide, Purified water, Synthetic spermaceti
	Blissel® vaginal gel	Estriol 50µg/g	Glicerol (E 422), p-hydroxybenzoic acid methyl ester, p-hydroxybenzoic acid propyl ester, Polycarbophil, Carbomer, Sodium hydroxide, Hydrochloric acid, Purified water
	Colpotrophine® vaginal cream	Promestriene 1g/100g	p-hydroxybenzoic acid methyl ester, p-hydroxybenzoic acid propyl ester, Mono and diglycerides of saturated fatty acids, Polyglycol ether of saturated fatty alcohols, Oleic acid decyl ester, Triglycerides of capric and caprylic acids, Glycerol, Purified water
Reference products	Universal placebo vaginal gel	Not applicable	Purified water, Hydroxyethylcellulose, Sodium chloride, Sobic acid, Caramel color, Sodium hydroxide
	Replens® Vaginal gel	Not applicable	Purified water, Polycarbophil, Paraffin oil, Glycerin, Palm oil hydrogenated, Carbomer homopolymer type B, Sorbic acid, Sodium hydroxide

Epithelial cells

The cell lines HEC-1A, HeLa and VK2 E6/E7 were obtained from the American Type Culture Collection (ATCC-LGC Promochem, Teddington, United Kingdom). The uterine HEC-1A cells, originated from a line of human endometrial adenocarcinoma (Kuramoto et al., 1972), were cultured in RPMI 1640 supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% FBS, further referred to as RPMI complete medium (Passages 33-38). HeLa cell line is also epithelial, derived from human cervical adenocarcinoma (Jones, 1997). These cells were cultured in DMEM F12 medium supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), and 10% FBS and is further referred to as DMEM complete medium (Passages 64-68). VK2 E6/E7 cell line is also an epithelial one, derived from human vagina (HPV-16 E6/E7 transformed) (Fichorova et al., 1997b). VK2 E6/E7 cells were cultured in Keratinocyte-serum free medium (Keratinocyte-SFM) added of 0.1 ng/mL human recombinant epithelial growth factor (hrEGF), 0.05 mg/mL bovine pituitary extract (BPE) and calcium chloride 44.1 mg/L (Passages 4-6).

Vaginal tissues explants

Complete porcine genitalia, from approximately 6 month-year-old animals, were collected from a local slaughterhouse, transported under refrigeration to the lab and processed within 3h of animal death. The vaginal tubes were separated from the surrounding organs using scissors and tweezers. The vagina was cut longitudinally and the vestibule (caudal vagina) excised. The vestibule was selected since we found (unpublished data) to be the proximal vagina histologically more similar to the human one. Tissues were then washed on a prewarmed saline solution (NaCl 0.9%, 37°C). This section of tissue was then thickness normalized by using a manual dermatome (Watson Skin Graft Knife, BBraun, Germany) equipped with Aesculap blades (BA718Rm, BBraun, Germany). Then, epithelial sheets were placed upon aluminium foil (contact made by the basolateral side) and punched to sections of $\varnothing=3\text{mm}$ using a biopsy punch (Stiefel, GSK, United Kingdom). These sections were maintained on a warmed saline solution until use in the MTT assay. Tissues thickness was accessed to maintain homogeneity between samples. For this purpose, a digital micrometer (Vogel, Germany) was used by placing the tissue between two microscopy slides, measuring total thickness (μm) and then subtracting the thickness of the slides to the total measured thickness.

Testing products preparation

Testing products were diluted to 20, 5, 1, 0.4, 0.2, 0.1% (w/v), in medium without supplementation but containing 0.5% (v/v) of DMSO to ensure proper solubility of formulations. DMSO was selected based on the recommendations of the ISO guide (ISO/EN10993-5, 2009). To assure that this substance was not itself cytotoxic, controls with the used concentration in culture media were performed. A negative control was included in all experiments (cells/tissues only with media/DMSO) and also positive controls for toxicity were included (SDS 5% (w/v), Triton X-100 1% (v/v) and N9 2% (v/v)). Formulations were left in contact with the testing platform (cell or tissue) for 24 hours.

Cellular toxicity

MTT assay

The MTT reduction assay was performed as previously described (Berridge et al., 2005; Denizot and Lang, 1986; Freshney, 2000; Plumb et al., 1989) and ISO/EN 10993-5 guide for *in vitro* evaluation of vaginal medical devices (ISO/EN10993-5, 2009). Cells were seeded onto 96-well plates (100.000 cells/mL) with the respective incomplete culture media. Cells were let to adhere for 24 hours at 37°C, 5% CO₂. After obtaining a half-confluent culture, 100 µL of testing formulations were added (see Testing products preparation section). After this period cells were washed with PBS and incubated for 4 hours with a 0.5mg/mL solution of MTT reagent prepared in the respective culture medium without supplementation. Subsequently to formazan crystals formation, extraction was accomplished with 200 µL of DMSO for 15 minutes on an orbital shaker protected from light. Absorbances were then measured at 590 nm and 630 nm for background deduction, using a microplate spectrophotometer (BIORAD XMark, USA). Additionally, cells were photographed before and after the application of the MTT reagent solution in order to evidence the formation of the formazan crystals, using an inverted microscope (Olympus IX51, Japan, equipped with OCTAX Eyeware v.1.5 Build 406, Germany) - data not shown. Triton X-100, SDS and Nonoxynol-9 were used as positive controls, since they are widely known inducers of cytotoxic effects (Cunha et al., 2014). The negative control consists of cells without any treatment (just culture media along the assay), which was considered as the 100% viability reference for products toxicity calculation. Further, the concentration that was toxic to half of the culture (cell/tissue), the half-maximal toxic concentration (TC₅₀), was calculated by logistic regression using GraphPad Prism Version 6.0 (Copyright, 2015).

NRU assay

The NRU assay was performed according to the literature (Freshney, 2000; Oecd, 2004; Repetto et al., 2008; Yang et al., 2008) and ISO/EN 10993-5 guide for *in vitro* evaluation of medical devices (ISO/EN10993-5, 2009). Cells were seeded into 96-well plates (100.000 cells/mL) and maintained in culture until a semi-confluent monolayer was achieved (37°C, 5% CO₂, 24 hours).

They were then exposed to the test compounds (100 μ L) over the range of concentrations described above. After 24 hours of exposure, wells were washed once with 150 μ L of prewarmed PBS, then 100 μ L of NR medium was added to each well and the plates incubated at 37°C in a humidified atmosphere of 5% CO₂ for 3 hours. After incubation, the NR medium was removed, and cells were washed with 150 μ L of PBS. Finally, 150 μ L NR desorb solution (ETOH/acetic acid) were added to all wells, including blanks, in order to extract the dye. Plates were rapidly shaken on a microtiter plate shaker for 10 min to allow NR to be extracted from the cells forming a homogeneous solution. The absorbance of the resulting coloured solution at 540 nm was measured in a microtiter plate reader (BIORAD XMark, USA). NRU was determined for each concentration and compared to the one determined in control cultures. For the study a NR stock solution was previously prepared by dissolving 0.4 g of the dye in 100 mL of milliQ water. The NR medium was prepared in the day prior of usage by adding 1 mL of NR stock solution to 79 mL of culture media, followed by incubation overnight at 37°C and filtration through a 0.2 μ m filter before adding to the cells, in order to be free of crystals. The NR desorb/extraction solution is composed of 1% glacial acetic acid solution, 50% ethanol and 49 % water.

Tissue toxicity

Method optimization

Tissue explants were firstly tested for homogeneity and response to MTT assay. Therefore, tissues were submitted to a preliminary experiment, using different animals and applying the viability test on fresh and frozen tissues. Frozen explants were obtained as described (section “Vaginal tissue explants”) but tissue preparation occur after defrosting; upon reception from the slaughterhouse the vaginas were opened longitudinally (from vulva to cervix), washed in a HBSS solution pH 4.2 and frozen at -20°C, wrapped in aluminium foil, and stored in airtight bags. For viability studies (fresh tissue: 46 animals, at least n=2 for each animal, 6 independent experiments; frozen tissue: 12 animals, n=3, one experiment), tissues were left in culture for 24 hours and then the MTT assay was performed. For toxicity studies (only performed on fresh tissue: 23 animals, at least n=2 for each animal, 5 independent experiments) tissues were put in contact with SDS 5% (w/v) during 24 hours, and then the MTT assay has begun. SDS 5% is generally used as a positive control in tissue toxicity assays and recognized to have a toxic effect even on epithelial vaginal tissue (Youssef Gali et al., 2010b).

MTT assay

Tissue explants were placed in 96-well flat-bottomed tissue culture plates and treated with the testing formulations diluted on RPMI media without supplementation at 37°C, 5% CO₂ during 24 hours, n=6. Also, negative controls were included for each animal. SDS 5% (w/v), Triton X-100 1% (v/v) and N9 2% (v/v) were used as positive controls. Subsequently, tissues were washed twice with prewarmed PBS. MTT solution at a final concentration of 0.5 mg/mL was added to each well and incubated 1 hour at 37°C, 5% CO₂. Tissues were then transferred into new plates and extracted with 200 μ L of isopropanol during 1.5 hours at room temperature under gentle

shaking. Finally, tissues were discarded for plate reading at 590 nm against the background at 630 nm (BIORAD XMark, USA). The tissue viability was calculated as percentage from the negative control (tissue with no formulation).

Histological analysis

One tissue per formulation, at its highest concentration, 20% (w/v), was reserved for histological analysis. Tissues were fixed in a balanced 10% formalin solution. Subsequently, fixed tissues were run in a set of ethanol solutions of increasing concentrations for dehydration before embedding in paraffin. Tissue blocks were cut to have about 3 μ m thickness and stained with haematoxylin and eosin. Slides were observed and microphotographs were taken using a Zeiss microscope (Axiomager A1, Zeiss, Germany) equipped with a digital camera (Axiocam, Zeiss, Germany).

Data processing and statistical analysis

Data was analysed to produce arithmetic means with standard deviations (SD) using Microsoft Excel. Analysis of variance (ANOVA) and Dunnett's multiple comparisons test were performed to determine the significance of the difference between sets of data ($p < 0.05$). Logistic regression analysis was performed using GraphPad Prism software to calculate the half-maximal toxic concentration (TC_{50}) in toxicity assays.

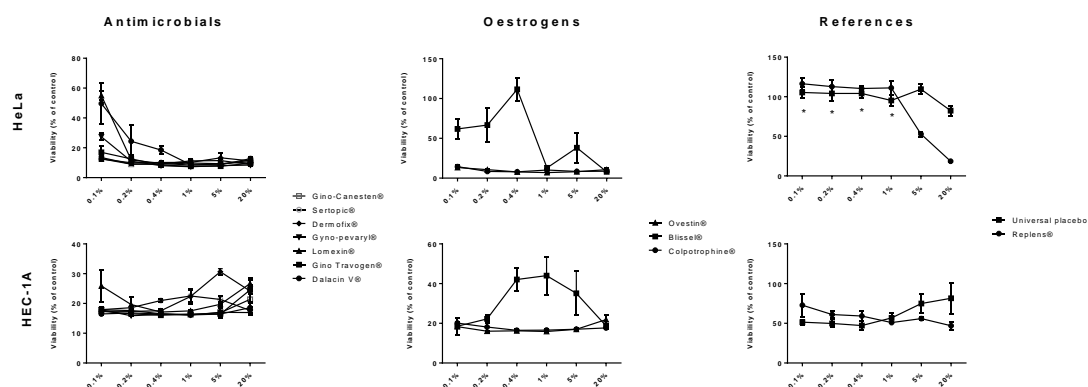
Results

Cellular toxicity

Cytotoxicity was accessed by means of the MTT reduction assay and NRU performed upon 3 different cell lines (HeLa, HEC-1A and VK2 E6/E7). Results are mean of 2 independent experiments, total n=6. All assays, were tracked microscopically for cell integrity, density and morphology, as stated in the respective protocol (ISO/EN10993-5, 2009). Cell cultures acquired half-confluency and were able to reduce MTT in the mitochondria, and to integrate NR in the lysosomes. Results are expressed as viability percentage of the negative control (cells treated only with media). For all experiments, positive controls were used to assure the occurrence of induced toxic effects. In the NRU assay viabilities for HeLa and HEC-1A cells, of the positive controls (Triton X100 1%, SDS 5% and N9 2%) were: 2.75 ± 0.26 ; 3.62 ± 0.278 ; 3.79 ± 0.30 ; 17.23 ± 0.91 ; 29.75 ± 9.85 ; $19.10 \pm 1.82\%$, respectively. Standard deviations for negative controls (cells with media) were 10.93 and 16.30% for HeLa and HEC-1A, respectively. The results for NRU assay using the VK2 E6/E7 are not showed because they were inconclusive. In the MTT assay viabilities for HeLa, HEC-1A and VK2 E6/E7 cells, positive controls had viabilities of (Triton X100 1%, SDS 5% and N9 2%): 2.75 ± 0.26 ; 3.62 ± 0.28 ; 3.79 ± 0.30 ; 5.82 ± 0.34 ; 5.86 ± 0.322 ; $6.54 \pm 0.50\%$, $26.52 \pm 1.45\%$; 13.35 ± 3.93 ; and 22.12 ± 2.17 , respectively. Standard deviations for negative controls were 10.93, 11.51 and 14.63% for HeLa and HEC-1A and VK2 E6/E7, respectively.

In **Figure 1** all profiles for cytotoxicity are represented both for the NRU and the MTT assay. As a general picture, HEC-1A and VK2 E6/E7 cell lines conducted to higher viabilities than HeLa cells, being higher in the NRU assay. Antimicrobials showed a linear decrease of viabilities along with the increasing of products' concentration, as expected. Furthermore, this behaviour occurred consistently on the three cell lines and within the two assays, exception made for antimicrobials studied in HEC-1A using the NRU assay, in which viabilities do not seem to be affected by the dilution factor.

A) NRU assay



B) MTT assay

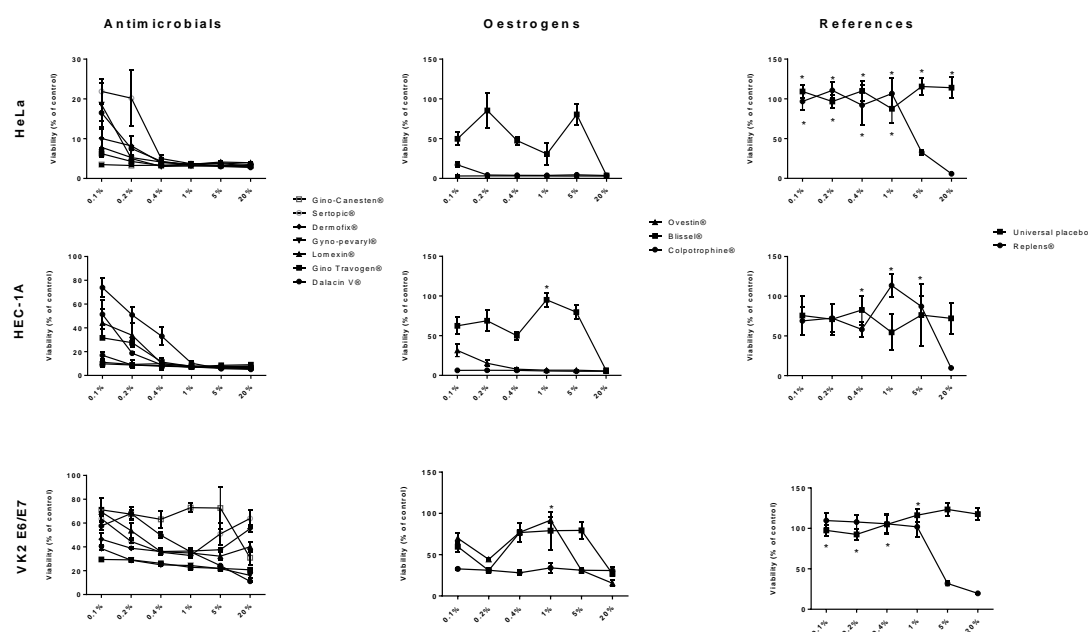


Figure 1: Cellular viability profiles for all the testing formulations at dilutions from 0.1 to 20% (w/v). Results for the NRU and the MTT assay. Viabilities are represented as percentage of the control treated only with culture media. Results are means and bars represent standard deviations from 2 independent experiments in which each condition was tested in triplicate (total n=6). * represents NO statistical difference from the control (two-way ANOVA, Dunnett's multiple comparisons test, $p < 0.05$).

The oestrogen-containing formulations, Ovestin® and Colpotrophine®, presented the higher extents of cell toxicity, independently of the tested concentrations (just a slight increase was observed in the 0.1% concentration). Profiles for the same cell line were concordant between the assays. Blissel® presented an odd profile, having a high increase on viability at 5%, which is reduced at intermediate concentrations, and then at low concentrations conducts again to high viabilities. This behaviour might be related with the Blissel® polymeric composition (polycarbophil/carbomer) which, in the lower concentrations promote lower toxicity and then promotes somehow a negative effect on cell proliferation at intermediate concentrations (actually this dilution could promote a better diffusion of estriol). Concerning the reference products, low cytotoxic profile was observed, as expected. Universal Placebo gel led to most

stable profile of viabilities across concentrations. Every concentration conducted to viabilities over 50% of cell viability. On the other hand, Replens® only showed to reach high viabilities at concentrations lower than 5%. This fact may be related with low pH presented by this formulation, around 2.8, which together with a high pH-buffering capacity and high osmolality, could explain the high level of cytotoxicity presented (Machado et al., 2017).

Regarding VK2 E6/E7 cells, there are no studies available on the literature presenting results in the NRU assay, despite these cells being largely used to study vaginal toxicity mechanisms through other methods. In fact, we found irregular results with these cells on the NRU method (n=3, 2 independent experiments) and this could be due to an increased NR uptake induced by lysosomal swelling agents such as weakly basic substances and by osmotic swelling agents such as polyols, as was demonstrated in previous studies (Ohkuma and Poole, 1981; Olivier et al., 1995). Taking this into account, the lysosomal swelling may lead to an underestimation of the cytotoxicity when the NR assay is used (Chiba et al., 1998; Repetto et al., 2008).

Tissue toxicity

Method optimization

Optimization experiments were performed to assure that after collection and cutting procedures, tissues would have enough viability to be used in a toxicity assay such as the MTT reduction assay. Also, this step resulted in better handling of the surgical instruments and consequently more precise cutting technique for the operator. Results for viability and toxicity studies are shown on **Table 2**. Tissue viability was performed either on fresh and frozen tissue. As expected, frozen tissues returned low absorbances, which are not adequate to a final MTT assay, since this would have been the maximum absorbance to obtain, and could not distinguish significant differences between formulations. For this reason, performing such toxicity studies in frozen-thawed tissues is not possible. Furthermore, the performance of a toxic substance (SDS) confirmed that chemicals can induced negative responses in *ex vivo* tissues. *One-way ANOVA* with Dunnett's multiple comparisons test was applied to assess differences between the fresh tissue, frozen tissue and tissue treated with SDS, ($p < 0.05$ was accepted as denoting significance). On the fresh tissue experiment it was clear that high absorbances can be reached using an *ex vivo* model, i.e. suitable for toxicity testing of formulations. Moreover, an acceptable variation coefficient can be held with our tissue preparation technique and culture procedure (24.39%).

Table 2: Optimization and pre-validation studies on tissues viability and toxicity. The viability study corresponds to a negative control (tissues plus culture media) and the toxicity study was performed using SDS 5%, recognized to have a toxic effect on epithelial vaginal tissue. *Denotes significance on One-way ANOVA Dunnett's multiple comparisons test ($p < 0.05$).

Viability studies (<i>Abs at 570nm</i>)		Toxicity studies (<i>Abs at 570nm</i>)
Culture media		SDS 5%
Fresh tissue (mean±SD)	Frozen (mean±SD)	Fresh tissue (mean±SD)
0.5788±0.1412	0.1015±0.0144*	0.0581±0.0133*
Variation Coefficient: 24.39%	Variation Coefficient: 14.20%	Variation Coefficient: 22.83%
46 animals	12 animals	23 animals
n=2-3	n=2	n=2-3
6 independent experiments	1 experiment	5 independent experiments

After measuring tissues thickness (6 animals, n=6, 3 independent experiments) the variation coefficient was calculated to be 15.12%.

MTT assay

Tissue viability results following exposure to the test products are shown in **Figure 2**. Only Universal Placebo and Ovestin® were found to conduct to viabilities >50% by the first dilution. All other formulations, except Dalacin V®, returned viabilities above 50% upon the second dilution (1:20). Standard deviations were relatively high, but acceptable considering that the surrogate used for this test is biological. Positive controls, Triton X100 1%, SDS 5% and N9 2%, conducted to viabilities of 3.22±0.77, 2.51±0.81 and 2.56±0.60 %, respectively, confirming that they have toxic effects on the vaginal epithelium (metabolic toxicity).

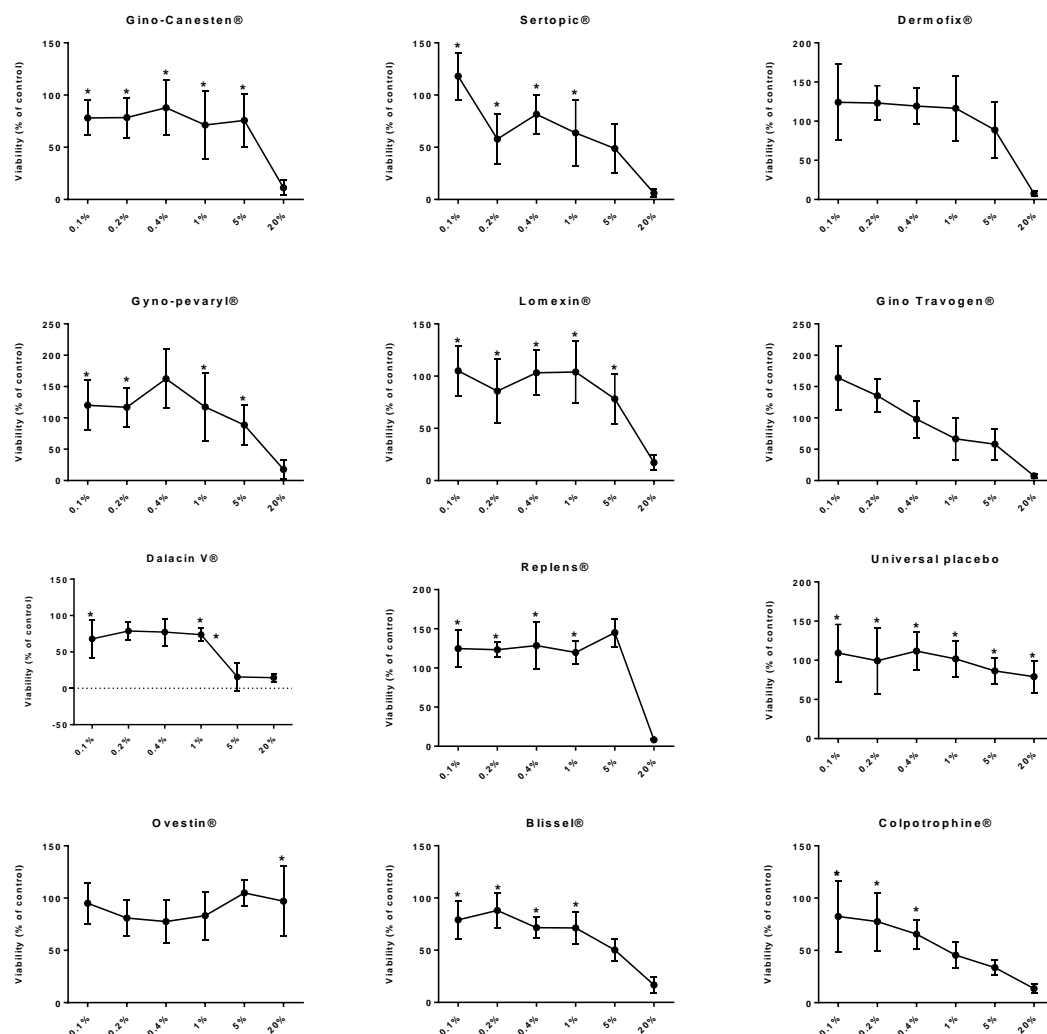


Figure 2: Tissue viability profiles for all the testing formulations at dilutions from 0.1 to 20% (w/v). Viabilities as percentage of the control tissue treated only with culture media. Results are means and bars represent standard deviations from 2 experiments in which each condition was tested in triplicate (total n=6). * represents NO statistical difference from the control (two-way ANOVA, Dunnett's multiple comparisons test, $p < 0.05$).

Histological analysis

Histological analysis performed on tissues after exposure to formulations was found to be a useful complement to tissue viability determination. Representative images of explants histology after exposure to the testing formulations are presented in **Figure 3**. All formulations seem to have induced epithelial alterations of the tissue when compared to the control. Nevertheless, oestrogenic (Ovestin®, Blissel®, Colpotrophine®) and reference products (Replens and Universal Placebo) were the ones to induce minimal changes. Also, Dalacin V®, an antibacterial, was able to preserve epithelial layers' integrity. On the other hand, antifungals showed extensive desquamation effects, being the most accentuated on Gino Canesten®. As expected, positive controls (Triton, SDS and N9) conducted an extensive damage

to epithelial layers. However, for Triton and N9 not as extensive as for SDS, which completely destroyed the epithelial and basal layers of the explant.

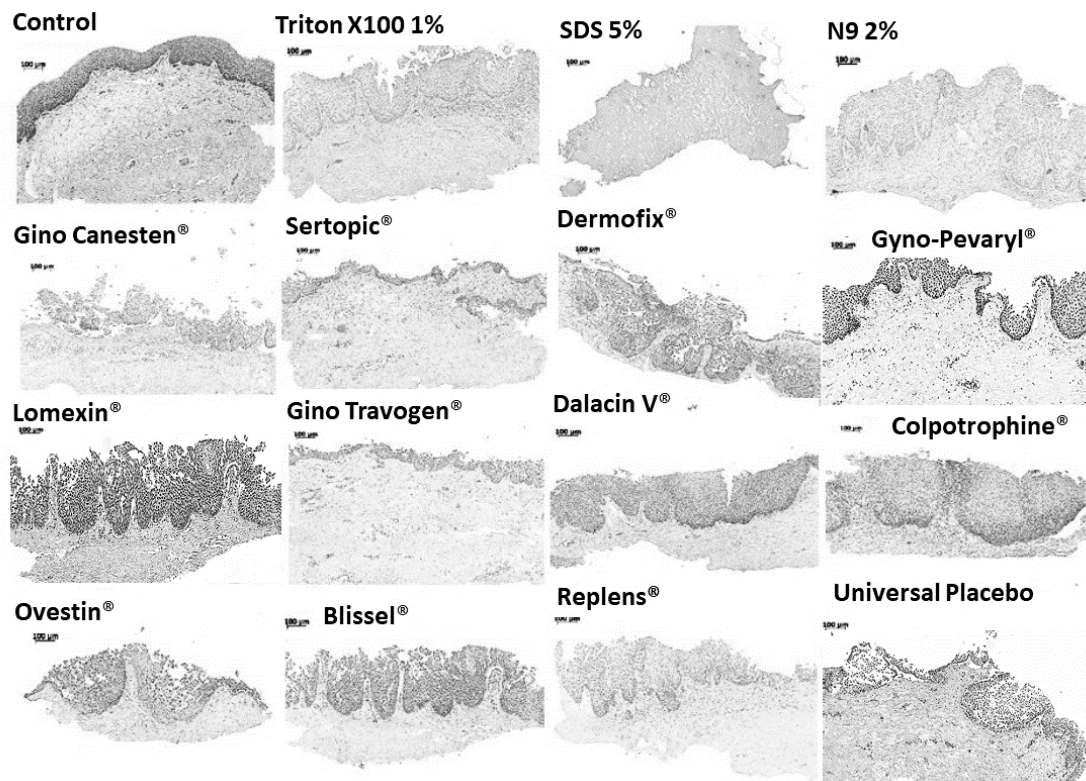


Figure 3: Impact of the tested formulations on the porcine *ex vivo* vaginal epithelium after 24 h of exposure. Histological images are representative of the higher concentration tested (20%), i.e. the worst-case scenario for the dilutions tested for tissue toxicity. H&E staining. Magnification 100x.

Models comparison

Calculated half-maximal toxic concentrations (TC_{50}) either for cellular models and tissue models are represented in **Table 3**. The *ex vivo* porcine vaginal tissue model, was the one that led to the calculation of the TC_{50} for almost all products. This means, on one hand that the formulations concentration in test is suitable to the test method, and on the other hand, that this method is the less sensitive in comparison to the remaining. Nevertheless, for Gino-Canesten® and Gino Travogen® the TC_{50} confidence interval was very wide, which could be related with the high slopes and SDs that are shown on the toxicity profiles for these products between concentrations 5 and 20%. The three cell lines were chosen because they were representative of three different epithelia that are present in the vaginal cavity, and, by testing these three cellular types we are addressing a complete toxicological profile of the formulation after administration by the vaginal route. Concerning the MTT assay, the most sensitive cell line was the HeLa (cervical cells), while VK2 E6/E7 (vaginal cells) was found to be the more robust. Since it is expected that cellular models are more sensitive to toxic effects than tissue models (due to the more complex and organized structure of the latter) and that the tissue, in turn, is more related to the *in vivo* environment, VK2 E6/E7 may provide more reliable results of the vaginal toxicity of formulations when performing the MTT assay. Neutral Red uptake assay should be performed to confirm previous results from the MTT assay, gathering the maximum data for preclinical safety characterization of formulations, or to circumvent problems due to chemical incompatibility. When possible, final formulations, that were selected to proceed for further preclinical and clinical stages, should be tested under an *ex vivo* model, being the technique herein present a valuable alternative test method.

Table 3: Half-maximal Toxic Concentrations (% - TC₅₀) calculated for cellular and tissue models using three different cell lines (HeLa, HEC-1A and VK2 E6/E7) and porcine vaginal explants. Within the cellular models, MTT and NRU assay were issued, as for tissues only MTT assay was performed.

Product	Cellular toxicity TC ₅₀ (%)			Tissue toxicity TC ₅₀ (%)		
	MTT assay			NRU assay		
	HeLa	HEC-1A	VK2 E6/E7	HeLa	HEC-1A	Porcine vaginal explants
Gino-Canesten®	<0.1	<0.1	7.039 (3.825-12.950)	<0.1	<0.1	18.530 (2.436-141.000)
Sertopic®	<0.1	<0.1	1.118 (0.550-2.272)	<0.1	<0.1	1.607 (0.568-4.549)
Dermofix®	<0.1	<0.1	5.243 (2.511-10.950)	<0.1	<0.1	>20
Gyno-pevaryl®	<0.1	0.196 (0.114-0.336)	<0.1	<0.1	<0.1	>20
Lomexin®	<0.1	0.232 (0.142-0.381)	0.296 (0.198-0.441)	<0.1	<0.1	>20
Gino Travogen®	<0.1	0.141 (0.114-0.173)	<0.1	<0.1	<0.1	11.950 (0.949-150.400)
Dalacin V®	<0.1	0.974 (0.420-2.256)	0.386 (0.302-0.495)	<0.1	<0.1	1.233 (0.628-2.422)
Ovestin®	<0.1	<0.1	2.163 (1.206-3.882)	<0.1	<0.1	<0.1
Blissel®	0.579 (0.288-1.167)	6.249 (2.953-13.220)	8.008 (3.293-19.480)	0.825 (0.436-1.561)	<0.1	3.030 (2.000-4.591)
Colpotrophine®	<0.1	<0.1	<0.1	<0.1	<0.1	0.911 (0.591-1.405)
Universal placebo	>20	>20	>20	>20	2.982 (0.722-12.320)	>20
Replens®	5.186 (2.203-12.210)	10.000 (8.877-11.260)	4.847 (3.270-7.186)	5.731 (3.851-8.529)	>20	>20

Discussion

The vaginal epithelium is a common route for topical administration of antimicrobials, oestrogens, lubricants, and hygiene feminine-care products (Alexander et al., 2004). Nonetheless, these products could potentially conduct to acute irritation of the epithelial surfaces. Since the vaginal cavity can be used for either sporadic or chronic product applications, special attention should be taken when designing new products for this route. Viral and bacterial infection potentiation subsequent to the use of some vaginal lubricants has been demonstrated, reinforcing the need of extensive safety characterizations both for medicinal and cosmetic/hygiene products (Adriaens and Remon, 2008; Begay et al., 2011; Cunha et al., 2014; Dezzutti et al., 2012; Maguire et al., 2001; Moench et al., 2010; Van Damme et al., 2002).

The standard preclinical test for the assessment of vaginal irritation and toxicity is the rabbit vaginal irritation (RVI) model. Similar to many other *in vivo* models, the reproducibility of results from this model (either between animals and between tests) is suboptimal. This variability is due to different responses of individual animals and to the trauma of insertion of the test product in the vaginal tract. Due to the high level of variability, standard RVI protocols use 10 rabbits per test article. The large number of animals required is undesirable from an animal welfare point of view and the variability decrease the confidence in the assay results. These problems are even magnified when primates are used for testing (Ayehunie et al., 2006).

Several *in vitro* and *ex vivo* models have been used to study the toxicity of substances and products, although it has not been stated which model better predicts *in vivo* toxicity (Youssef Gali et al., 2010a). On one hand, *ex vivo* assays are closer to fully represent the *in vivo* mechanisms. On the other hand, *in vitro* techniques are easier to handle, quicker, more sensitive and able for high-throughput screening in early steps of discovery and development. During product development, it has been observed that even slight modifications in final formulations, can either modify the efficacy and/or toxicity of a vaginal preparation (Y Gali et al., 2010; Goeman et al., 1995).

Therefore, in our toxicity assays we assessed the toxicity profiles of final formulations belonging to different therapeutic classes (antimicrobials, oestrogens and reference products). The formulations included in this study are already commercialized and of course regarded as safe for human use, but our main purpose was to provide scientists involved in formulation with a rational tool that could rapidly disclose final products toxicity before getting to animal studies. Having that in mind, we were expecting that these finished products could, despite being safe, present some sort of *in vitro* toxicity. This actually means that poor results in *in vitro* stages of safety testing do not have to conduct to similar results *in vivo*. Some of the results herein present are somehow not clearly explained because the authors did not have access to the fully quantitative information of the formulations. This is the case of Blissel®, that presented an odd profile, although consistent in the two tests, models, and cell lines, a profile which is

thought to be due to the presence of estriol. The behaviour presented is actually similar to the one that generally is seen for oestrogens *per se*. In fact, Blissel® is the oestrogenic formulation with the lower concentration of oestriol (50µg/g vs 1mg/g in Ovestin® and Colpotrophine®), and since the concentration in these last two is considered high, that might explain the cytotoxic profile over all formulation dilution (even at 0.1%, the estriol content is able to saturate all oestrogenic receptors conducting to oxidative stress) (Bhat et al., 2003). Additionally, Blissel® showed an oestrogen-driven behaviour, conducting high cellular viabilities in low concentrations and low viabilities at high concentrations. Moreover, it was clear that the cellular models were affected by dilution effects (inconsistencies after certain dilutions), which do not represent a methodological issue in our *ex vivo* model. These findings along with the difficulties in attributing the toxic actions to a single substance, let us state that this screening of final formulation should be proceeded by individualized excipient and active substances responses. A study conducted by Gali et al (2010) exposed the toxic effects of various excipients for the vaginal route belonging to different classes, and the results obtained offer a useful guidance to select the most promising excipients displaying their toxicity *in vitro*. A criteria for selection appointed by the authors is that the concentration of a specific excipient should be below the CC₅₀ (Youssef Gali et al., 2010c). Transposing that assumption to this work we could estimate the product concentration on the vaginal fluid at each moment - 0.75 mL (Owen and Katz, 1999) and, adjust the recommended doses in order to minimize toxicity, although warranting the therapeutic effect. Likewise, Gali et al., we found interesting correlations between the data obtained in the various assays. This implies that the different assays do not generate independent data, although the sensitivities can differ. Therefore, to begin with screening, the simplest assay should be preferred. Thereafter, the most promising formulations could then be confirmed in a more relevant model, such as the *ex vivo* model, and after that an *in vivo* model.

It is well established that data obtained using *in vitro* models during the preclinical stages of development will not match entirely the outcomes in clinical trials. This happens because, firstly, the settings are different on the two stages (API concentration and length of exposure and, of course, the biological environment), and, secondly, the parameters evaluated in clinical trials are wider. Such as, histopathological evaluation, assessment of the vaginal inflammatory condition, pH and microflora appreciation. Consequently, while there is no certainty about the *in vivo* standard for safety assessment, potential safety issues detected on *in vitro/ex vivo* assays should be seriously considered. Several international research groups and organizations have reunited efforts to identify vaginal biomarkers, including cytokines and chemokines, in an attempt to correlate *in vitro* and *in vivo* safety testing more properly. Fichorova et al. in 2001, have established a correlation between mucosal toxicity and increased levels of the proinflammatory chemokine IL-8 in vaginal washings of spermicide-treated rabbits (Fichorova et al., 2001). Thus, quantification of IL-8 might be used as a sensitive analyte to complement *in vitro* toxicity testing.

It is clear that before beginning product evaluation, all excipients and APIs should be checked for biocompatibility. The product evaluation comes next to reinforce the non-toxic effects after the formulation step. Testing semisolids formulations (i.e. final formulations) toxicity using both *in vitro* and *ex vivo* models has previously been performed. For example, Rohan et al. showed that a tenofovir gel and a placebo gel composed of hydroxyethylcellulose, EDTA, citric acid, glycerin, and the preservatives methyl- and propylparaben were detrimental toward epithelial cells and explants, causing a reduced viability and epithelial layer integrity of HEC-1A and Caco-2 cells and cervical tissue explants (Rohan et al., 2010). Similar observations were made by Dezzutti et al., assessing the toxicity and epithelial layer integrity of HEC-1A and Caco-2 cell lines after exposure to PRO-2000 gel, UC-781 gel, and the placebo gels methylcellulose and Vena Gel (Dezzutti et al., 2004). Furthermore, our workgroup has already tested several vaginal lubricants commercially available, under a safety perspective, concerning cytotoxicity, pH and osmolality (Cunha et al., 2014). Earlier a similar study has been performed by Dezzutti et al. on 10 aqueous-based lubricants to test not only cellular toxicity and damage to epithelial monolayers, but also their toxicity upon human explants epithelium. Main findings of toxicity to the microflora were attributed to preservatives contained in the formulations. Alongside, it was concluded that epithelial trauma alone would not be sufficient to increase the HIV-1 transmission, having inflammatory response an important role in this (Dezzutti et al., 2012). Similarly, to the study herein presented these authors refer that they had some difficulties in performing the toxicity tests in the cellular models, because, when testing creams (which are composed of an oily phase and an aqueous phase) it was difficult to solubilize and wash the cells during the tests. Actually, we have circumvented this problem by using 0.5% (v/v) of DMSO in the final formulation dilution with culture media to prevent phase separation of these type of products in test. Furthermore, with handling practice and constant microscopy monitoring, we were able to check for cell detachment after washing steps (which we concluded that do not happened). Still, it was also observed that washing steps were even easier when performing the tests on the *ex vivo* model.

In 2006, MatTek™ Corporation, an American enterprise specialized in developing and producing organotypic models, i.e. reconstructed tissue models for drug/product testing concerning several routes of administration, published an *in vitro* vaginal human tissue model with low variation coefficients intra and inter-batch (<10 and <15%, respectively), being an innovative and improved toxicological test system (Ayehunie et al., 2006). In comparison to *in vivo* models, this system (EpiVaginal™) reduces the testing duration, allows for high-throughput screening, and complies with the “3Rs” policy leading to the decrease of number of animals used to screen feminine-care products. Besides, it could be applied to vaginal drug delivery assays, bacterial adhesion and omics (Sanjay Garg, Kaustubh R. Tambwekar, Kavita Vermani and Chaman L. Kaul, 2001). EpiVaginal™ is available as an epithelial tissue (grown from normal human vaginal epithelial cells) and as a full thickness tissue (including epithelial cells and a fibroblast-containing lamina propria). These two tissues could also include immune-competent dendritic cells for inflammatory studies. Another company providing researchers with this type of tools

in Europe is EpiSkin™, producing HVE (reconstructed human vaginal epithelium) that is composed of A431 cells (derived from a vulvar epidermoid carcinoma). It is well established that these tools are clearly advantageous when in comparison with other *in vitro* and *ex vivo* models, not only in terms of sensibility and reproducibility, but also in terms of technology, quality control and even technical assistance. Nevertheless, they have main applicability to industry screening platforms, rather than academic research, because of the economical expenditure. That is a key point why *ex vivo* tissues could represent valuable tools for scientific research, when early stages of the preclinical development of drugs/products is the focus. Moreover, the *ex vivo* models take into consideration an interindividual variability that is not present in manufactured reconstructed models, and that in fact, is closer to the variability encountered further on *in vivo* studies.

Despite having a full cell structure (epithelial, connective, immune) and better tolerance to formulations, tissue explants also have some drawbacks like variability, and the fact that they are technically more demanding. Also, *ex vivo* tissue is not entirely representative of the *in vivo* situation due to lack of tissue regeneration, lack of immune cells recruitment, and independence from hormones (Dezzutti et al., 2012; Rohan et al., 2010). If human explants are used, they can also be of limited number and require an Institutional review board approval (Gorodeski, 1996).

In our study, a good overlapping profile could be found between the cellular and the tissue model, although cellular testing was hypersensitive, which conducted to lower viabilities, and even making impossible the calculation of TC₅₀. Indeed, the ability to calculate TC₅₀ for almost all formulation in the *ex vivo* model indicates that this model is valuable to assure direct comparisons, even allowing the use of more concentrated dilutions of formulations, if experimentally possible.

The *ex vivo* porcine tissue model herein presented extends the use of *ex vivo* porcine tissue as a surrogate for human vaginal tissue. There is a great deal of data that validate the porcine model of vaginal mucosa in terms of structure, function and reactivity in comparison to human tissue. Both have stratified squamous epithelium supported by connective tissue. The use of small, *ex vivo*, specimens provides convenience, efficiency, and high throughput for screening (Machado et al., 2015; Squier and Mantz, 2008). Samples of porcine tissue are inexpensive to obtain (in abundance on slaughterhouses) and handling is easy when compared to the use of whole animals (Costin et al., 2011; Squier and Mantz, 2008). *Ex vivo* porcine tissues have been largely tested for drug permeation. But, despite appearing to be a good *ex vivo* permeability model for human vaginal tissue extrapolation it has already been shown that its reliability may vary upon substances chemical characteristics. For example, for hydrophilic molecules, (water and vasopressin, for example), the porcine vaginal tissue is an accurate *in vitro* permeability model of human tissue, while for more lipophilic molecules (such as oxytocin) the flux could be higher than the corresponding estimated value for human tissue (van Eyk and van der Bijl, 2005). Another limitation related to the *ex vivo* vaginal model is the fact that some authors

use cervical and uterine explants, to resemble the vaginal epithelia (Cummins et al., 2007; Youssef Gali et al., 2010c; Merbah et al., 2011; Rohan et al., 2010). This may be due to the difficulty in accessing human vaginal epithelium and/or also based on the assumption that vaginal formulations shall be safe not only to the vagina but also to the uterus. The porcine model can relatively circumvent this problem of accessing specimens for testing and provide a better similitude with the human vagina itself. Added to that, tissue collection and culture procedures had not been standardized and optimized, before this research. Previous studies of ours have been focused on disclosing the likeliness of porcine vaginal tissue and the human tissue, and even disclosing which anatomical region of the porcine vagina is more appropriate to collect in order to comply with human similarity (in preparation for publication).

The tetrazolium-based MTT assay has long been regarded as the gold standard of cytotoxicity assays as it is highly sensitive and has been miniaturised for use as a high-throughput screening assay. The first use of the MTT assay upon tissue explants goes back to the 90's, when it was applied to different types of tissues, for instance buccal mucosa (Imbert and Cullander, 1999; Radosevich et al., 1993). This method is also the one recommended by the manufacturers in the EpiVaginal™ and HVE™ reconstructed models. Recently, Van Tonder et al. in 2015, conducted a study comparing several toxicity assays, including the MTT and NRU assays in MDA-MB-231, MCF-7 and MCF-12A cell lines. Results indicated that the NRU showed one of the smallest variability across the linear range, while the largest variation was observed for the MTT assay. This implies that this assay would more accurately detect small changes in cell number than the MTT assay. Furthermore, the MTT assay was the one to have more interferences with test products (van Tonder et al., 2015). Although seeming less sensitive and with higher number of interferences, MTT has a large historical use, and researchers are familiar with the methodology. Furthermore, standard documents like those from the "International Organization for Standardization", specifically the (ISO/EN10993-5, 2009) recommend this procedure for cytotoxic assessment of medical devices. Moreover, it has a successful history of application in tissues.

Conclusions

In vitro and *ex vivo* models have been applied to assess the safety profiles for drugs, excipients and final formulations in order to early preview toxicity issues that might arise in the ensuing steps of product development. Herein we presented some preclinical toxicity results obtained for a panel of vaginal semisolid formulations, already regarded as safe for human use. Furthermore, we have not only optimized a strategy to perform *in vitro* cellular toxicity assays using semisolid aqueous and non-aqueous formulations; but also, developed a collecting technique for vaginal *ex vivo* porcine tissues. The later showed to be a possible strategy *for ex vivo* toxicity testing, since it demonstrated high reproducibility and sensitivity, with low variability. The inclusion of this method in preclinical safety assessments may optimize cost-efficiency of new formulations development by predicting efficacy and safety profiles at early stages of product development. It could not only be applied to the pharmaceutical industry and research, but also to the cosmetic, hygiene, and medical devices industries. Moreover, it could further be applied to the development of primary cell cultures, as a surrogate for permeation and metabolic studies; and to originate co-cultures with microorganisms.

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APPENDIX C

Research article that contributed to the second experimental part of CHAPTER III in the format that has been submitted.

Research Article

“Testing vaginal irritation with the HET-CAM assay: an *in vitro* alternative to the *in vivo* method”

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Summary

The HET-CAM (Hen's Egg Test-Chorioallantoic Membrane) assay is an *in vitro* alternative to the *in vivo* Draize Rabbit Eye test which mimics vascular changes in the chorioallantoic membrane. It consists on a qualitative method to assess the irritancy potential of chemicals. The CAM responds to injury with an inflammatory process similar to the one observed in the conjunctival tissue of a rabbit's eye. Regarding topical toxicity assessment of medical devices, it has been stated that, the sensitivity of eye and vaginal epithelium is similar (ISO 10993-5). Consequently, the aim of this work was to apply the HET-CAM assay to test the irritancy potential of vaginal formulations. Vaginal semisolid medicines and lubricants currently marketed were tested along with the Universal Placebo formulation which safety has been clinically shown. Nonoxynol-9 (N-9), a known vaginal irritant, was enrolled as positive control (concentrations ranging from 0.001 to 100% (v/v)). The assay was conducted according to the ICCVAM - Recommended test method (NIH Publication No. 10-7553 - 2010). Formulations were then classified according to its irritation score (IS), using the analysis method (A) and (B). The studied vaginal formulations showed low potential for irritation. N-9 was classified as a severe irritant at concentrations above 2%, which corroborates clinical data from the literature, envisaging a possible *in vitro/in vivo* correlation. IS (B) was considered as a better classification output. Although still requiring further validation, the HET-CAM assay seems an ideal prospect for vaginal irritancy potential *in vitro* studies.

Keywords: vaginal irritation, HET-CAM, *in vitro*

1. Introduction

Topical toxicity has been a main topic within European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM). Having also a regulatory responsibility, the EU and associated laboratories, have been working on developing and validating toxicity test methods for eye irritation, phototoxicity, skin corrosion, irritation and sensitisation (European Commission). Furthermore, several *in vitro* Test Guidelines are already provided or being developed by the Organisation for Economic Co-operation and Development (OECD). *In vitro* studies have been valued and recommended as a first screening for toxicity evaluation, in detriment to *in vivo* models.

The assessment of irritability and toxicity of products for vaginal administration does not yet include *in vitro* methods validated and recognized by both the referred authorities. Currently, there are numerous medicines and medical devices marketed or being in the development phase for vaginal administration. (Woolfson et al., 2000; Alexander et al., 2004; Hussain and Ahsan, 2005; Choudhury et al., 2011). The parallelism between sensitivity to eye and vaginal irritants is addressed in the ISO 10993 guideline since eye irritant products are included in the exclusion criteria for vaginal irritation test, being considered irritant to the vagina (ISO 10993-5, 2009). The HET-CAM assay is already widely applied to ocular application products, yet its possible application for the testing of vaginal formulations is an innovative proposal.

As a general assumption, maximum effectiveness with the least amount of adverse effects is always desired for vaginal formulations. Since these products are mostly for self-administration and should offer maximum comfort both at the moment of application and during the time of usage, effects such as local irritation become especially important to be evaluated. One of the methods most used to assess vaginal irritation is performed *in vivo* on rabbits (RVI) (OECD; Eckstein et al., 1969). However, the European Commission and REACH (Registration, Evaluation and Authorization of Chemicals), have joined efforts in pursuance of the 3Rs policy (Replacement, Reduction, Refinement) and do prohibit marketing of cosmetic products tested in animals (European Commission, 2003). The HET-CAM assay is one of the alternatives presented by ECVAM for the *in vitro* study of ocular irritability (ICCVAM, 2007; European Commission). Although various techniques for the characterization of vaginal formulations have been described on the literature (Garg et al., 2001, 2010; Adriaens and Remon, 2008; Cunha et al., 2014), most have been applied only to developing formulations with no correlation with *in vivo* results. The HET-CAM assay outcome consists on an Irritation Score (IS) that is a value calculated by different analysis methods (A and B), which is used to classify the irritancy potential of a test substance. The IS (A) analysis method takes into account the observation of endpoints at specified time points after application of the test substance (0.5, 2, and 5 min post exposure). At the time points, the presence of an endpoint is determined and a score assigned, in case it is present. The scores are calculated to yield an overall irritation score. Instead, when applying the IS (B) analysis method, the endpoints are monitored over the entire

observation period after applying the test substance (typically 5 minutes). The time (in seconds) when an endpoint develops is registered and these values are used to yield an overall irritation score using a mathematical formula (U.S. Public Health Service, Department of Health and Human Services, 2006).

In this study, our aim was to evaluate the possibility to use the HET-CAM assay as an *in vitro* alternative method to the *in vivo* vaginal irritation test. Vaginal semisolid medicines and lubricants were examined for their irritation potential considering two scoring categories, IS (A) and IS (B). Additionally, nonoxynol-9, a well-known vaginal irritant substance, was studied from concentrations ranging from 0.001 to 100% (w/v).

2. Materials and Methods

2.1. Chemicals and testing products

For the preparation of the assay controls, the following chemicals were used: sodium chloride (NaCl, JT Baker, USA), sodium dodecyl sulphate (SDS, Acros Organics, Belgium), nonoxynol-9/tergitol (N-9, Sigma, Germany), sodium hydroxide (NaOH, VWR Prolabo, Germany) and type I water (Millipore, Merck, USA).

The testing products included in this study were vaginal semisolids, available in the international market, intended both as therapeutics for several pathological conditions, and as lubricants for sexual and menopausal discomfort. The ten different therapeutic products were: Gino-Canesten® (Bayer), Sertopic® (Ferrer), Dermofix® (Azevedos Laboratories), Gynopevaryl® (Johnson & Johnson), Lomexin® (Jaba Recordati), Gino Travogen® (Bayer), Dalacin V® (Pfizer Laboratories), Ovestin® (Aspen Pharma), Blissel® (ITF Medivida), Colpotrophine® (Teva Pharma). The lubricants tested were: Fillergyn® gel (BSDpharma), Geliofil® Classic gel (Laboratoires Effik), GelSea® gel (LDPSA), Ginix® gel (ISUS), Ginix® Plus gel (ISUS), Hyalo Gyn® gel (Fidia Farmaceutici), K-Y® Jelly (Johnson & Johnson), Phyto Soya® gel (Arkopharma Laboratoires Pharmaceutiques), Velastisa® Intim VG moisturizer gel cream (ISDIN) and Vidermina® gel (Istituto Ganassini). Both Replens® (Laboratoires Majorelle) and Universal Placebo (Tien et al., 2005) were used as controls, since their toxicity profiles are largely described on the literature (Nachtigall, 1994; Bygdeman and Swahn, 1996; Tien et al., 2005; Valenta, 2005; Schwartz et al., 2007; Adriaens and Remon, 2008; Acartürk, 2009; Garg et al., 2010; Clark et al., 2011; Caramella et al., 2015). Universal Placebo was prepared by dissolving of 2.7g of Hydroxyethyl-cellulose (2000cP) in 96.3g of water containing 0.85g of sodium chloride and 0.1g of sorbic acid. The final pH was adjusted to 4.4 by adding sodium hydroxide, and the gel was stored at 2-8°C. To evaluate the method sensitivity to vaginal irritants, nonoxynol-9 was used in concentrations ranging from 0.001 to 100% (v/v).

2.2. Eggs and incubation conditions

The test system consists on Leghorn white hen's white eggs, fresh (not more than 7 days old), clean, weighing 45 to 65 g. When the eggs arrived to the lab, they were checked for damages in the shell: damaged eggs were discharged while the others were incubated, at $37.8 \pm 0.3^\circ\text{C}$ in an atmosphere with a relative humidity of $58 \pm 2\%$ and under automatic rotation, during 8 days (Corti AF-50 and Copele 30652, Spain). By the eighth day, eggs were inspected using a LED light to confirm the embryo formation. Non-embryonated eggs were discarded and the others were incubated for 1 one more day in the same conditions but without egg rotation.

2.3. HET-CAM assay

The assay was conducted according to the ICCVAM - Recommended test method (NIH Publication No. 10-7553 - 2010). At day 9, the eggs were taken out of incubator and were placed on an

appropriate support with the larger part facing up. The shell was opened with the help of a scalpel and tweezers and the chorioallantoic membrane (CAM) was exposed. This membrane was then hydrated with NaCl 0.9% (w/v) for a maximum of 30 min. Afterwards the solution was aspirated and the membrane was peeled off without damaging the blood vessels. For each product 0.3 mL were applied on the membrane and 3 eggs per products were used. The irritant effect of these products was evaluated by monitoring the appearance of three endpoints in the CAM, for 5 minutes: haemorrhage (vessel bleeding), lysis (vessels disintegration) and coagulation (protein denaturation intra and extra- vascular). In the present study, these endpoints were evaluated accordingly to two different criteria: Irritation Score (IS) A and B. While for criteria A the endpoints were checked at predetermined time intervals (0.5; 2 and 5 min), for criteria B these effects were monitored continuously during 5 minutes paying attention to the time when the irritant response begun which was registered. This methodological difference conducts to different range of categories in the irritant outcome (see **Table 1**). Photographs were taken at the beginning and end of the assays. Calculation of the IS for each test product is represented as mean \pm standard deviation (SD) of a total of three eggs.

3. Results

The irritation potential determined for N-9 using the HET-CAM assay was reasonably comparable when using both evaluation tools, IS (A) and (B), as it can be observed in **Figure 1**. Only at the concentration of 0.5% (w/v) the difference between these two testing criteria results was statistically different (two way-ANOVA, $p < 0.05$, Sidak's multiple comparisons test), although standard deviations were relatively high. This concentration seems to be the one that presents a *borderline*-type behaviour, since its score (on criteria B) is in the limit between slight and moderate irritant. The same might be happening for concentration 1% which is in between moderate and severe irritant, on the IS(B) scale. It can be also observed that concentrations above 2% were the ones that gave IS values higher than 9, meaning that at these concentrations N-9 exhibits severe vaginal irritation effect (IS (A) $N-9\ 2\% = 10.0 \pm 0$; IS (B) $N-9\ 2\% = 10.8 \pm 0$). Taking into consideration the IS (B) criteria, concentrations between 0.3 and 1% were classified as moderately irritants (IS (B) $N-9\ 0.3\% = 4.8 \pm 0.5$; IS (B) $N-9\ 1\% = 8.7 \pm 0.4$). Concentrations between 0.01 and 0.2% were slight irritants (IS (B) $N-9\ 0.01\% = 3.7 \pm 0.6$; IS (B) $N-9\ 0.2\% = 4.9 \pm 0.7$). The two lowest concentrations tested, 0.001 and 0.005%, were regarded as non-irritants, having scores of 0.9 ± 0.8 and 1.1 ± 1.0 , respectively. In what concerns to the IS (A) criteria, the non-severe irritant response was found for concentrations ranging from 0.001 and 1 with scores of 0.7 ± 0.6 and 7.3 ± 1.2 , respectively. These results show that the most irritant N-9 concentration is found independently of the scale being used. Nonetheless, the IS (B) is able to discriminate more irritation categories when compared to IS (A).

The irritation scores determined for the vaginal formulations are represented on **Figure 2**. Concerning the therapeutic products **Figure 2 (a)**, Universal Placebo, Replens®, Dermofix®, Sertopic®, Dalacin V®, Ovestin® and Blissel® did not conduct to any irritant response, having been scored with 0 ± 0 . Gyno Pevaryl®, Gyno Canesten® and Colpotrophine® had significantly different scores when evaluated with the two criteria. Concerning criteria A, they were all classified as non-severe irritants. However, when the criteria B was used, Gyno Canesten®, Colpotrophine®, Gyno Pevaryl® were classified as slight irritants (IS (B) $Gyno\ Canesten\text{®} = 3.3 \pm 0.3$; IS (B) $Colpotrophine\text{®} = 2.0 \pm 0.0$; IS (B) $Gyno\ Pevaryl\text{®} = 4.8 \pm 2.0$). All except Gyno Travogen®, had scores higher in scale B than on scale A (IS (A) $Gyno\ Travogen\text{®} = 3.0 \pm 0.0$; IS (B) $Gyno\ Travogen\text{®} = 2.0 \pm 0.6$), although this difference was not statistically supported.

Regarding the vaginal lubricants (**Figure 2 (b)**), only Phyto Soya® had a score of zero. Hyalo Gyn®, Velastisa VG® and Gelsea®, which showed no significant difference when comparing both scales, also obtained the least irritant scores. The remaining products had significant differences between the evaluation of IS (A) and (B). The remaining products had significant differences between the evaluation of IS (A) and (B). All lubricants were classified as non-severe irritants by the IS (A) irritation criteria with Ginix® reaching the highest score in this scale (3.7 ± 1.2). When the IS (B) was applied Hyalo Gyn® and Velastisa VG® were classified as non-irritants and all the others were classified as slight irritants. For all but Ginix®, the score

obtained in scale B was higher than the one obtained in A. As it can be seen on **Figure 2 (b)**, when comparing both scales, they also obtained the least irritant scores. The remaining products had significant differences between the evaluation of IS (A) and (B). **Table 2** details the score obtained and the classification attributed to all products tested in this study. Furthermore, a picture from the test end-time (5 min) is also shown for products and controls. As it can be observed, some products did not present the same classification when comparing the two scales (*borderline* behaviour). In the case of medicines, Gyno Pevaryl®, Gino Canesten®, Lomexin®, Gino Travogen® and Colpotrophine®, were the ones to present a classification of slight irritants on IS (B) and non-severe irritants on IS (A). Regarding lubricants, KY Jelly®, Vidermina®, Ginix Plus®, Geliofil® and Fillergyn®, presented this behaviour.

4. Discussion

Nowadays, the increasingly number of new chemicals and products introduced into the market coming from several industries (cosmetic, pharmaceutical and medical devices) has generated a need to validate *in vitro* techniques able to screen potential irritation effects at the early stages of development.

The Hen's Egg Test, or Hühner-Embryonen-Test (HET), firstly presented by Luepke, back in the 80's, was developed to be a rapid, sensitive and inexpensive toxicity test able to provide information on embryotoxicity, teratogenicity, systemic, metabolic and immunopathological effects (Luepke, 1985). This method was designed to be applied in mucous-membrane irritation testing, and the tool provided for score and classification was analogous to the Draize test. Since then, it has been confirmed that a good correlation between these two tests, i.e. *in vitro/in vivo* correlation, for plenty of chemicals do exist. The HET-CAM assay cannot totally replace the irritation tests in mammals, but can largely decrease the number of animals used, and limit the pain and injury inflicted to animals during experiments (Luepke, 1985).

The application of the HET-CAM assay to vaginal irritation testing comes in line with the referred compliance with the 3R's policy. In the vaginal products field, there is no organotypic irritation test approved neither on the EU, nor the USA. In fact, there are some cellular and tissue models available for toxicity testing, however mainly comprise techniques for specific metabolic pathways, histological analysis and inflammatory response (Fichorova et al., 2004; Repetto et al., 2008; Gali et al., 2010; Costin et al., 2011). Our research group is focused in developing strategies for preclinical safety characterization of vaginal products using cellular and *ex vivo* tissues assays (paper submitted for publication). The usage of HET-CAM assay in vaginal products testing comes to widen the safety assessment portfolio that can be applied to test substances or products in a quicker and more effective way on the first step of preclinical safety testing. In this study, several semisolid vaginal medicines and lubricant products were tested. Moreover, two analytic methods of the IS were applied. It was concluded that when testing N-9, a pure substance, there was no statistical difference when applying one analytic method or another. Actually, the concentration found to be the one that could trigger severe irritant effects, 2% (w/v), was the same that was shown to generate severe toxicity in clinical trials, being less safe than thought at preclinical evaluations (Van Damme et al., 2002). In that case, N-9 was being studied as a spermicide, but, because of its surfactant nature, it ended up on being irritant, and even promoting the transmission of HIV infection (Stafford et al., 1998; Phillips et al., 2000; Dayal et al., 2003). Having this episode in mind, the need for more appropriate *in vitro* assays to predict *in vivo* safety issues, is even more highlighted. In our study, the surfactant nature of N-9 may be responsible for the fact that when increasing abruptly its concentration (up to the pure substance) no higher rate of irritation was observed (U.S. Public Health Service, Department of Health and Human Services, 2006). Universal placebo, on the other hand, was herein included because it is a vaginal gel that was wittingly

design to be a control formulation in clinical trials of vaginal microbicides (Tien et al., 2005; Schwartz et al., 2007). Its safety profile, already confirmed by clinical trials, was once more confirmed in this study, having an IS of zero on the two scoring grids (A and B).

Concerning semisolid vaginal medicines, no severe irritant responses were observed, and this outcome was already expected. They are already commercialized and since they are classified by the competent authorities as medicines, they were not only subjected to preclinical evaluations, but also to extensive clinical trials. Moreover, the products herein classified as non-severe irritants (IS (A)) and slight to moderate irritants (IS (B)), such as Gyno Pevaryl®, Gino Canesten®, Colpotrophine®, Gino Travogen® and Lomexin® were also tested by our research group using two *in vitro* cytotoxicity tests: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and neutral red uptake (NRU) into uterine (HEC-1A), cervical (HeLa) and vaginal (VK2 E6/E7) cell lines; and, an *ex vivo* porcine vaginal model using the MTT reduction assay and histological analysis; having also demonstrated preoccupant toxicity profiles. In fact, Gino Canesten® had one major impact on the vaginal *ex vivo* epithelia when histological analysis was performed (paper submitted for publication). Having this in mind, and concerning our cytotoxicity results, it was also herein demonstrated that these products could be considered *borderline* products, which means they have different classifications when using one scale or another. Moreover, Gino Travogen® and Colpotrophine®, were also the ones that presented higher toxicities on a toxicity *ex vivo* model that we developed. This data coincidence might corroborate the need to perform several *in vitro* tests for a complete toxicity evaluation, since the combined results might be stronger predictors for *in vivo* toxicity, as the current mind-set in toxicology states (Samuel et al., 2016). Furthermore, we also performed some technological characterization of the same products, having safety in perspective. Accordingly, we determined the osmolality, since it represents a predictor for irritation (Adriaens and Remon, 2008) and found that Lomexin® and Colpothrophine® were highly hyperosmolal (1446 ± 20 and 1723 ± 20 mOsmol/kg, respectively), above the upper limit recommended by the WHO (1200 mOsmol/kg) (World Health Organization, 2012; Machado, 2017). In fact, isosmolality is well known to be an important technological parameter for safety of both ocular and vaginal products. Previously, we performed a similar study on vaginal lubricants and found that Geliofil®, Vidermina®, K-Y® Jelly, the ones that showed higher IS in the HET-CAM assay, had extremely high osmolalities (3582 ± 11 ; 3707 ± 16 and 3631 ± 13 mOsmol/kg, respectively). Besides, Ginix and Ginix plus were also the ones that showed higher toxic profiles on HeLa cervical cells using the lactate dehydrogenase (LDH) colorimetric cytotoxicity assay (Cunha et al., 2014). Once again, K-Y® Jelly and Ginix Plus®, that were considered as HET-CAM *borderline* products, had previously demonstrated higher cytotoxicities. These findings, strengths the hypothesis that products osmolality and cellular and tissue toxicity could be highly predictive of irritation potential and, it can further suggest that these techniques (HET-CAM, osmolality, cell/tissue metabolic toxicity) should be applied concomitantly for a more robust clinical irritation response preview.

The application of the two scoring analytic methods, IS (A) and (B), confirmed that IS (B), although being more difficult to perform and also requiring a more qualified operator, can conduct to better classification output. Also, it leads, generally, to higher IS which means it could be scoring false positive irritants, rather than false negatives. In a safety perspective, this should be regarded as valuable in comparison to IS (A).

The suitability of the HET-CAM assay for vaginal irritation testing was demonstrated with this study, despite more assays and controls are needed to be enrolled to complete the validation process as well as inter-laboratory testing in order to confirm its reproducibility. Additionally, the improvements that were tested in the past for the HET-CAM applied to testing cosmetic ingredients eye toxicity, like an additional histological analysis (Djabari et al., 2002) and the combination of two softwares (ImageJ® and Adobe Photoshop®) that allows live monitoring of the assay, reducing the subjectivity in the endpoints evaluation (McKenzie et al., 2015) shall be considered in this approach in order to assure more accurate results.

The HET-CAM assay has already been applied to test the irritation potential in other epithelia. In 1999, Lönnroth et al tested eight polymeric products to be used as dental restorative materials (Lönnroth et al., 1999). Later, on 2007 other research group inquired the irritative potential of dental adhesive agents, and answered this question by performing the HET-CAM assay (Dahl, 2007). Furthermore, it has been applied to testing of skin irritation, for the anti-inflammatory response of plasma to treat chronic skin wounds (Bender et al., 2011), and also, to evaluate the irritation potential of topical antiseptics (Marquardt et al., 2010). The HET-CAM has also been applied to test medicines for ocular application, for example compounded fluconazole and voriconazole eye drops prepared in an hospital pharmacy department to disclose potential eye irritation (Fernández-Ferreiro et al., 2014).

Although using incubated Hen's eggs for tests could represent a borderline case between *in vivo* and *in vitro* systems, it does not conflict with ethical and legal obligations especially animal protection laws. It was already demonstrated that incubation up to day nine, the embryonic differentiation of the chicken central nervous system is sufficiently incomplete to avoid suffering and pain perception. Actually, the few sensory fibers present at day nine only develop after incubation during 11 to 14 days (Liebsch et al., 2011). Studies also suggested that the extraembryonal vascular systems (e.g., yolk sac, CAM) are not sensitive to pain (Spielmann, 1995). Therefore, this test method can reduce the number of animals subjected to testing and reduce the pain and suffering of rabbits by their exclusion from the testing of corrosives and severe irritants (U.S. Public Health Service, Department of Health and Human Services (2006)).

Until now no single *in vitro* test has emerged as being completely acceptable for full replacement of *in vivo* tests. However, the Hen's Egg Test Chorioallantoic Membrane has gained regulatory acceptance in various countries to classify severe eye irritants, and has potential to be applied to other mucosal/epithelial substrates such as the vaginal epithelia.

5. Conclusions

The HET-CAM assay was successfully applied to vaginal irritation testing. This strategy represents an innovative approach for the preclinical safety assessment of vaginal products, being them classified as medicines, cosmetics, hygiene products or medical devices. The application of the two scoring analytic methods, IS (A) and (B), confirmed that IS (B) can conduct to better classification output and preferably should be chosen. The studied vaginal formulations, comprising medicines and lubricants, showed, as expected, low potential for irritation. N-9 was considered as a severe irritant above 2% (w/v) concentrations, which corroborates clinical data from the literature, envisaging a possible *in vitro/in vivo* correlation. Comparisons with previous studies by our workgroup confirmed that HET-CAM can predict and/or confirm toxic profiles for products also tested for osmolality and cellular/tissue toxicity. Ideally, an integrative methodology should be designed to embrace all these preclinical tests, for a better *in vivo* safety preview. Although still requiring further validation, the HET-CAM assay seems an ideal prospect for vaginal irritancy potential *in vitro* studies.

6. References

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Conflict of interest

The authors declare that they have no conflicts of interest.

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Figures

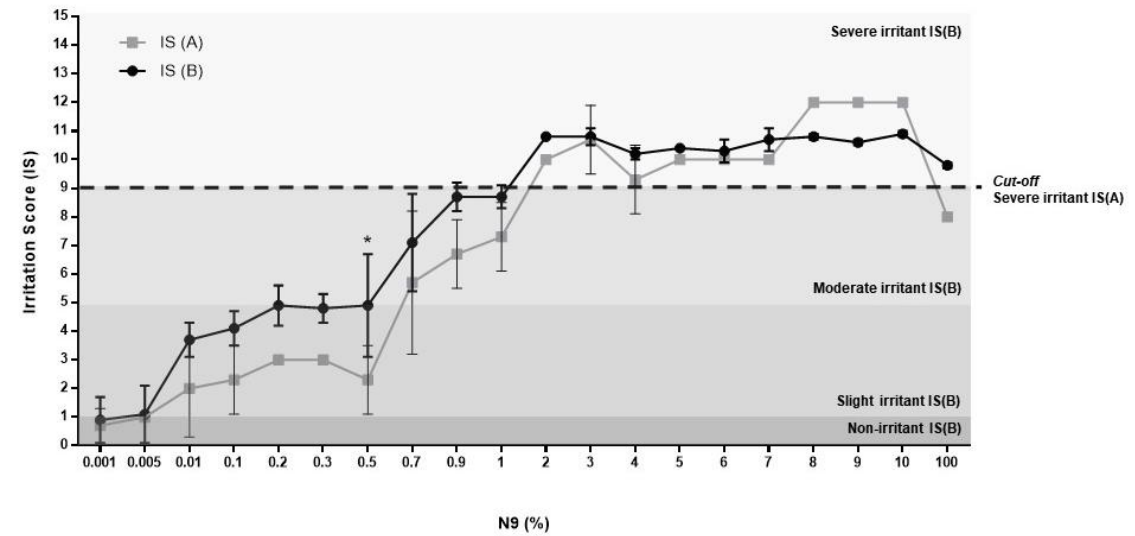


Figure 1: Irritation Scores (IS) for N-9 (nonoxynol-9) according to categories A and B. Results are presented as mean values \pm standard deviation (SD), $n=3$. * denotes statistical difference between the two scales IS (A) and IS (B) (two way-ANOVA, $p < 0.05$, Sidak's multiple comparisons test).

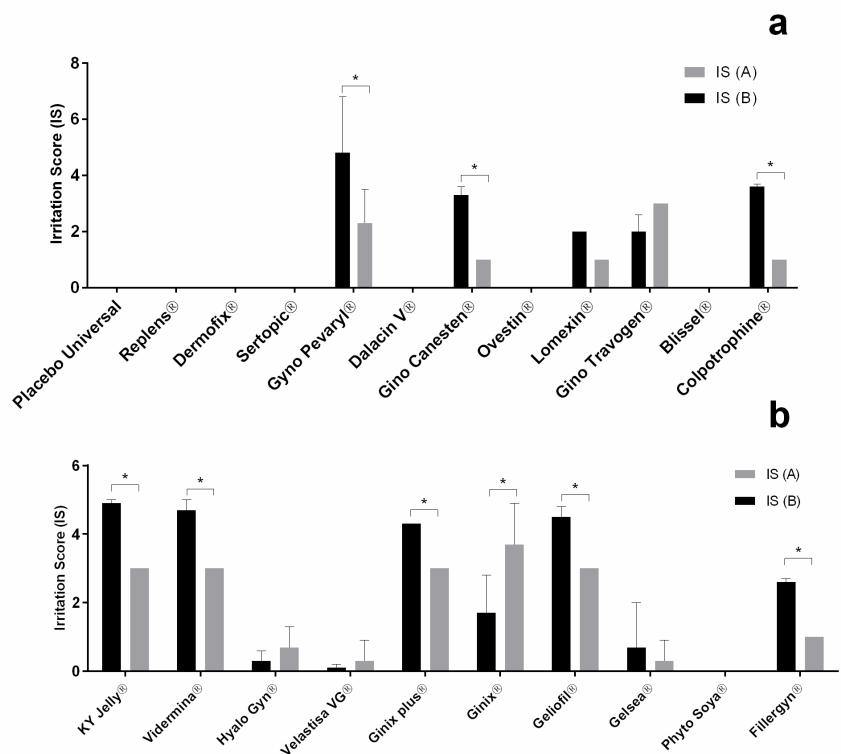


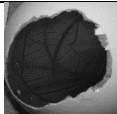
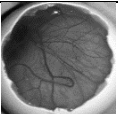
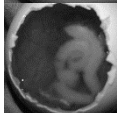
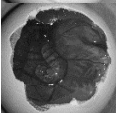
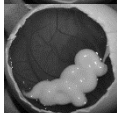
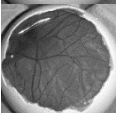
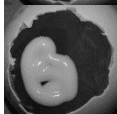
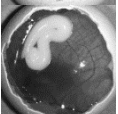
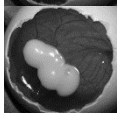
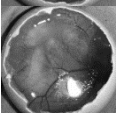
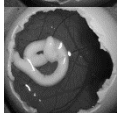
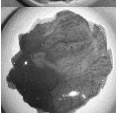
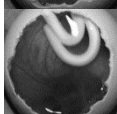
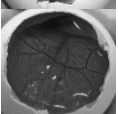
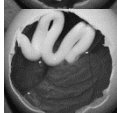
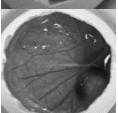
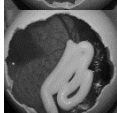
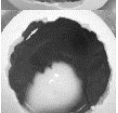

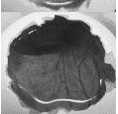
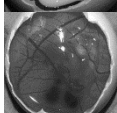
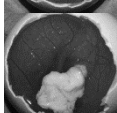
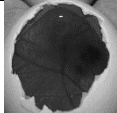
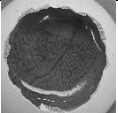
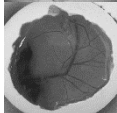
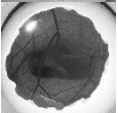
Figure 2: Irritation Scores (IS) for therapeutic vaginal products (a) and vaginal lubricants (b) according to the categories A and B. Results are presented as mean values \pm standard deviation (SD), $n=3$. * denotes statistical difference between the two scales IS (A) and IS (B) (two way-ANOVA, $p < 0.05$, Sidak's multiple comparisons test).

Tables

Table 1: Irritancy classification. Classification on the *in vitro* HET-CAM assay concerning Irritation Score (IS) analysis methods A and B.

Irritation Score (IS) analysis methods A and B.																								
Analysis method A																								
Irritation response		0 to 8: Non-severe irritant 9 to 21: Severe Irritant																						
IS calculation method		Calculation of the IS is the sum of the scores attributed at each time point to the arising of the corresponding effect, as stated on the following scheme: <table><tr><th>Endpoint</th><th colspan="3">Score</th></tr><tr><td></td><td>0.5 min</td><td>2 min</td><td>5 min</td></tr><tr><td>Lysis</td><td>5</td><td>3</td><td>1</td></tr><tr><td>Hemorrhage</td><td>7</td><td>5</td><td>3</td></tr><tr><td>Coagulation</td><td>9</td><td>7</td><td>5</td></tr></table>			Endpoint	Score				0.5 min	2 min	5 min	Lysis	5	3	1	Hemorrhage	7	5	3	Coagulation	9	7	5
Endpoint	Score																							
	0.5 min	2 min	5 min																					
Lysis	5	3	1																					
Hemorrhage	7	5	3																					
Coagulation	9	7	5																					
Analysis method B																								
Irritation response		0 to 0.9: Non-irritant 1 to 4.9: Slight Irritant 5 to 8.9: Moderate Irritant 9 to 21: Severe Irritant																						
IS calculation method		Calculation of the IS by applying the following equation: $\left[\left(\frac{(301 - Hemorrhage\ time)}{300} \right) \times 5 \right] + \left[\left(\frac{(301 - Lysis\ time)}{300} \right) \times 7 \right] + \left[\left(\frac{(301 - Coagulation\ time)}{300} \right) \times 9 \right]$ <p>Times should be replaced by the time (in seconds) at which each effect started.</p>																						

Table 2: Irritation potential. Irritation scores, IS (A) and (B) determined for vaginal semisolid medicines and lubricants.

Medicines				Lubricants			
Product		IS (A)	IS (B)	Product		IS (A)	IS (B)
Placebo Universal		Non-severe irritant (0)	Non-irritant (0)	KY Jelly®		Non-severe irritant (3)	Slight irritant (4.9)
Replens®		Non-severe irritant (0)	Non-irritant (0)	Vidermina®		Non-severe irritant (3)	Slight irritant (4.7)
Dermofix®		Non-severe irritant (0)	Non-irritant (0)	Hyalogyn®		Non-severe irritant (0.7)	Non-irritant (0.3)
Sertopic®		Non-severe irritant (0)	Non-irritant (0)	Velastisa VG®		Non-severe irritant (0.3)	Non-irritant (0.1)
Gyno Pevaryl®		Non-severe irritant (2.3)	Slight irritant (4.8)	Ginix plus®		Non-severe irritant (3)	Slight irritant (4.3)
Dalacin V®		Non-severe irritant (0)	Non-irritant (0)	Ginix®		Non-severe irritant (3.7)	Slight irritant (1.7)
Gino Canesten®		Non-severe irritant (1)	Slight irritant (3.3)	Geliofil®		Non-severe irritant (3)	Slight irritant (4.5)
Ovestin®		Non-severe irritant (0)	Non-irritant (0)	Gelsea®		Non-severe irritant (0.3)	Slight irritant (0.7)
Lomexin®		Non-severe irritant (1)	Slight irritant (2)	Phyto soya®		Non-severe irritant (0)	Slight irritant (0)
Gino Travogen®		Non-severe irritant (2)	Slight irritant (3)	Fillergyn®		Non-severe irritant (1)	Slight irritant (2.6)
Blissel®		Non-severe irritant (0)	Non-irritant (0)				
Colpotrophine®		Non-severe irritant (1)	Slight irritant (3.6)				
Assay controls							
Product		IS (A)	IS (B)	Product		IS (A)	IS (B)
N-9 2% (v/v) (Positive control)		Severe irritant (10)	Severe irritant (10.8)	NaOH 0.1N (Positive control)		Severe irritant (19)	Severe irritant (19)
SDS 1% (w/v) (Positive control)		Severe irritant (10)	Severe irritant (10)	NaCl 0.9% (w/v) (Negative control)		Non-severe irritant (0)	Non-irritant (0)

APPENDIX D

Literature review tables that supported the design of the experimental setup of CHAPTER IV:

- The first table details already published methods for drug quantification using HPLC-DAD.
- The second demonstrates the theoretical predictive calculations for molecules concentration in the receptor chambers.
- The third table shows a comparison for receptor media for *in vitro* drug release and *ex vivo* permeation experiments already published on the literature.

Bibliographic references used in this literature review are listed below the respective table.

HPLC-DAD methods for the permeation products

Molecule	HPLC-DAD method	Permeation method	Concentration expected to permeate	Reference
CLOTRIMAZOL 10mg/g	<p>Waters HPLC-DAD Detection: 233nm Colum: stainless steel C-18 reverse-phase column packed with 5µm particles (Waters, XTerra RP-18, 3.9mm × 150mm) Mobile phase: methanol/water (75:25, v/v) Flow rate: 0.5mL/min Injection volume: 50µL.</p>	<p>Franz diffusion cells: To evaluate CTZ permeation through the skin, samples of pig mucosa were mounted on standard Franz diffusion cells (area=1.0±0.1cm²) and the receptor compartment (volume 11mL) was filled with PBS. After equilibration, 1mL of CTZ-NLC or CTZ-NLC-gel was added to the donor compartment. The receptor compartment was stirred at 250rpm in a thermostatic water bath at a temperature of 37°C. At fixed time intervals up to 8h, 100µL of the receptor phase were withdrawn and replaced with an equal amount of fresh PBS. The samples were filtered with 0.20µm hydrophilic PVDF. Tissues extraction: the mucosa was rinsed twice with ultrapure and stored at -19°C for 1h. Tissues were cut in vertical slices and collected in glass vials. CTZ penetrated into slices was extracted with 2 mL of methanol under overnight stirring with magnetic bars. The obtained samples were filtered with 0.20µm.</p>	<p>Loaded concentration: CTZ 1mg/mL and it is not expected to permeate. Toxicity: hepatic and can induce resistance especially in patients with a compromised immune system (Liu et al., 2010; Tettenborn, 1974). The results showed that no CTZ was found in the receptor medium even after 8 h of diffusion. The results showed that only 0.33±0.06% of the CTZ applied by CTZ-NLC was found within the mucosa (0.0033mg/mL).</p>	(Ravani et al. 2013)
	<p>Beckman HPLC Colum: Beckman C18 5µm, 4.6mm×25cm. Mobile phase: methanol and 0.1 M phosphate buffer (pH 3) (27:73). Flow rate: 1mL/min. Detection: 254nm. This method was validated in terms of specificity, linearity and reproducibility. The limit of quantification was 0.1µg/mL. Recovery: 90.1-94.2%</p>	<p>Franz diffusion cells: receptor compartment had a volume of 7.2 mL and an effective diffusion area of 0.785 cm² (ø=0.5cm). The receptor compartment was filled with a PBS solution containing 10% (v/v) dioxane which was constantly stirred with a magnetic bar (37±1°C). Frozen rabbit vaginal mucosa. Samples of 200µL were withdrawn from the receptor at specified time intervals and replaced with an equal volume of fresh receptor solution. In addition, the vaginal mucosa was cleaned on both sides and the drug accumulated in the mucosa was extracted with 100% ethanol and subjected to five sonication cycles of 30 min each in an ultrasound bath. The concentration of drug in the receiver at various times and that extracted from the mucosa was determined by HPLC. Control solution was 1% clotrimazole (w/v) PEG 400.</p>	<p>Loaded concentration: 1.5 mg of drug Solubilisation enhancers: 0.1M citrate phosphate buffer (pH5.5) containing 10% Tween-80; 0.1M acetate buffer, pH6 and organic dioxane (35%v/v); PBS (pH7.4) solution with 10% dioxane.</p>	(Ninga-b et al. 2005)
	Other HPLC methods (without the permeation application)			
	<p>Waters 1500 Series HPLC Colum: C18 reversed octadecyl phase 250mm × 4.6mm, 5m. Mobile phase: ACN-MeOH-0.15% phosphoric acid pH 7.5 (15:60:25V/V/V) Flow rate: 1.0mL/min. Detection: 270nm Injection volume: 50 µL Temperature: 25°C (Sehati et al. 2015)</p>	<p>Shimadzu LC-2010C system Stock solutions of individual chemicals (active ingredient and internal standard) were prepared in acetonitrile. The reference standard solutions were prepared diluting the respective stock solutions to the final concentrations of CLO (500mg/L), ibuprofen (100mg/L), IM (5mg/L) and CDM (5mg/L) The stock solution of internal standard (ibuprofen) was prepared dissolving 500mg in 100 mL of acetonitrile All solutions were at 4°C. Colum: Zorbax SB-Phenyl column (75mm×4.6 mm, 3.5µm) Mobile phase: acetonitrile and water in ratio 65:35 (v/v) and pH=3.5 (adjusted with 85% phosphoric acid) Flow rate: 0.5 mL/min IBU was used as the internal standard Injection volume: 5µL Detection: 210 nm at ambient temperature Run time: 6 min</p>	<p>Merck Hitachi HPLC Colum: C18 stationary phase (250×4.0m) bonded onto 5µm silica gel manufactured by Merck Flow rate: 1.5 mL/min Injection volume: 5µL Detection: temperature ambient and 254nm Standard stock solution: 0.5 mg/mL of clotrimazole Mobile phase: Acetate buffer with a pH of 6.8 was prepared by dissolving 25.0g ammonium acetate in 1000 mL of distilled deionised water. Diluent was prepared by mixing 990 mL of methanol and 10 mL of acetic acid. (Manassra et al. 2010)</p>	(Hájková et al. 2007)

SERTACONAZOL	20mg/g	Waters e2795 HPLC Detection: 225 nm Mobile phase: acetonitrile:disodium phosphate buffer (pH 3) (60:40, v/v) Column: Luna C18 (5µ, 250x4.6mm) at 40°C Flow rate: 1.0 mL/min Run time: 12 minutes and the retention time of the drug was around 5.6 min. Standard solution: 100 µg/mL was prepared by dissolving the drug in PEG:PBS (60:40) which is the receptor medium that will be used for <i>in vitro</i> studies. (Manian et al.)		Column: 10-µm Spherisorb CN Mobile phase: acetonitrile and aqueous 0.01 M sodium phosphate (37:63, v/v). Detection: 260 nm at 35°C, the retention time being 19.3 min. Injection volume: 25µL Flow rate: 1.6 mL/min (Albet et al. 1992)		
ECONAZOLE	10mg/g	Column: Zorbax C8 column. Mobile phase: methanol:water containing 0.06 mL triethylamine pH 10 (75:25 v/v) Flow rate: 1.5 mL/min Detection: 225 nm (Mousa et al. 2008)	HPLC-DAD Varian 5020 Detection: 270nm, ambient temperature Column: Hypersil Mobile phase: methanol-0.05 M TEA phosphate buffer (pH 7.0) (85:15, v/v) Flow rate: 1 mL/min (Di Pietra et al.)	HPLC Varian 5020 Detection: ambient temperature, 230 nm Column: 5-km reversed-phase Nova-Pak RP- 18 column (150 x 3.9 mm, i.d.) Mobile phase: using a mobile phase of methanol-tetrahydrofuran-0.1 M triethylammonium acetate (pH 7.0) (70:12:18, v/v/v) Flow-rate: 0.8 mL/min (Cavrini et al. 1989)	HPLC Hewlett Packard Column: RP-18 column (100x8 mm, 4/µm) (Waters) Flow rate: 1 mL/min Detection: 260 nm Mobile phase: acetonitrile-10 mM:ammonium acetate (65:35; v/v) with 0.5% diethylamine Run time: 30 min (Ng et al. 1996)	Column: RP-18 Mobile phase: methanol/aqueous ammoniumcarbonate solution/tetrahydrofurane (Christinat & Zulliger 1984)
FENTICONAZOLE	20mg/g	(fenticonazole was used as internal standard) Column: C8 reversed-phase Mobile phase: methanol-0.005 M tetrabutylammonium dihydrogenphosphate (77:23, v/v) Flow rate: 1.0 mL/min Detection: 270 nm Retention time: 10.3 min (Han et al. 1997)				
ISOCONAZOL	10mg/g	HPLC-DAD Varian 5020 Detection: 270nm, ambient temperature Column: Hypersil Mobile phase: methanol-0.05 M TEA phosphate buffer (pH 7.0) (85:15, v/v) Flow rate: 1 mL/min (Di Pietra et al.)		HPLC Thermo FINNIGAN, Column: C18 (100 mmx4.6 mm, thermoquest) Mobile phase: acetonitrile and 0.05 M Ammonium Acetate buffer in the ratio of 70:30 (v/v pH 6.0) Detection: 254 nm at ambient temperature Flow rate: 1.0 mL/min (Dayyih et al. 2012)		
ESTRIOL	1mg/g (Ovestin) 50µg/g (Blissel)	Waters 2695 HPLC Column: Alltima C18 column Flow rate: 1.0 mL/min Mobile phase: methanol-water (62:38, v/v) Detection: at 221nm (Shi et al. 2008)	Agilent 1200 HPLC Column: Agilent SB-C18 reversed-phase column (4.6x250mm id, 5 mm particle size) Detection: 25°C, 205 nm Mobile phase: 3.0mM ammonium acetate solution and acetonitrile organic modifier Flow rate: 1.0 mL/min Injection volume: 20µL (Wang et al. 2009)	Waters HPLC Column: Agilent Zorbax Eclipse XDB C8 (5 µm, 250 mm by 4.6 mm) Detection: 230 nm Flow rate: 1.0-mL/min Mobile phase: linear gradient from 30% acetonitrile and 70% water to 80% acetonitrile and 20% water over 30 min. (Hubinge)		

HPLC-DAD Agilent 1100

Colum: Kinetex hexyl-phenyl column,
150x4.6 mm, 2.6 µm particle size

Mobile phase: mixture of water (A) and ACN (B); at 0 min is based
on 40% of B and following the next gradient composition: 0-15 min
60% (B), 15-19 min 60% (B), 19-22 min 40% (B), 22-25 min 40% (B) (25
1C)

Flow rate: 0.5 mL/min

Injection volume: 10 µL

Detection: 200 and 240 nm
(Almeida & Nogueira 2015)

Agilent 1100

Colum: Zorbax Eclipse XDB-C18 (150x4.6 mm, dp =5µm)

Detection: 27°C 280 nm

Mobile phase: 100 mM phosphate buffer, pH
4.6/methanol/acetonitrile (50:20:30%, v/v)

Flow rate: 0.8 mL/min

(Sadowski & Gadzała-Kopciuch 2013)

1200 LC system

Colum: Eclipse XDB-C18 column (4.6x150mm,5µm)

Detection: 30°C

Mobile phase: acetonitrile/water (55:45, v/v)

Flow rate: 0.8 mL/min

Injection volume: 20µL

(Hu et al. 2013)

Predictive calculations for molecules concentration in the receptor chamber (assuming 100% and 1% permeation)
200-400mg of testing formulation→ calculation for 200mg (minimum)

CLOTRIMAZOL	SERTACONAZOL	ESTRIOL (Ovestin)
10mg/1000mg 2mg/200mg	20mg/1000mg 4mg/200mg	1mg/1000mg 0.2mg/200mg
2mg/15mL→0.13mg/mL (100%) 1.3µg/mL (1%)	4mg/15mL→0.27mg/mL (100%) 2.7µg/mL (1%)	0.2mg/15mL→0.013mg/mL (100%) 0.13µg/mL (1%)
ECONAZOLE	FENTICONAZOLE	ESTRIOL (Blissel)
10mg/1000mg 2mg/200mg	20mg/1000mg 4mg/200mg	0.05mg/1000mg 0.01mg/200mg
2mg/15mL→0.13mg/mL (100%) 1.3µg/mL (1%)	4mg/15mL→0.27mg/mL (100%) 2.7µg/mL (1%)	0.1mg/15mL→6.7µg/mL (100%) 0.067µg/mL (1%)
ISOCONAZOL		
10mg/1000mg 2mg/200mg		
2mg/15mL→0.13mg/mL (100%) 1.3µg/mL (1%)		

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COMPARISON OF RECEPTOR MEDIA

	<i>In vitro</i> drug release	<i>Ex vivo</i> permeation
CLT	<ul style="list-style-type: none"> The analyses were carried on using Franz type glass diffusion cell assembled with a nylon membrane (GNWP, 0.2 µm pore size, Millipore, MA). The cell body was filled with a receptor phase constituted of an ethanol:water mixture (20:80, v/v). (Esposito et al. 2013) To investigate the formulation's stability in simulated physiological condition, the drug amount retained in vesicles change of liposomes/niosomes and corresponding vesicles gel were tested in simulated vaginal fluid (SVF) (pH 4.2) at 37±1°C for 24 hr. (Ning et al. 2005) The <i>in vitro</i> release of clotrimazole in free form and in complex with B-cyclodextrin was determined from different vaginal gel formulations using a dialysis bag placed in a sealed glass vial under constant magnetic stirring. The gel formulations (2.5 g) were packed into the dialysis bags Spectra/Por Cellulose EsterMembraneMWCO: 100 000 Da, Spectrum Labs, Rancho Dominguez, CA) sealed with closures of 50 mm (Spectrum Labs). The release medium was 100 mL 0.1M citrate-phosphate buffer (pH 5.5) containing 1% Tween 80, providing sink conditions for clotrimazole. (Bilensoy et al. 2006) Release assays were performed in a Hewlett Packard automatic dissolution testing connected to a Prolabo Dissolutest apparatus (method no. 2, USP 23rd Ed.). The dissolution medium was a litre of phosphate buffer (pH 7.2). (Blanco-Fuente et al. 2002) Release kinetics of model drugs from amphiphilic B-CD nanospheres (1 mg) were determined in 20 mL of Water:PEG 400 (60:40) providing sink conditions in a thermostated shaker bath system (Memmert, Schwabach, Germany) at 37°C. (Memişoğlu et al. 2003) <i>In vitro</i> release profiles of CMZ-MBG and Candid-V® were studied using modified USP XXIII apparatus I at 37±0.5° C with a rotating speed of 25 rpm in buffer pH 4.5 citrate phosphate buffer as a dissolution medium. A watch dish containing 1.0 g of the developed formulation was tightly secured with a stainless steel wire screen (350 µ mesh size sinker). (Bachhav & Patravale 2009) A volume of 50 mL Sorenson's phosphate buffer (pH 7.4) containing 1% sodium lauryl sulfate (SLS) maintained at 37°C and stirred at a speed of 100rpm was used as the release medium. (Hashem et al. 2011) Dissolution media 0.1M citrate-phosphate buffer (pH 5.5) containing 1% Tween 80. (Yun Chang et al. 2002) Solubility: Solubility studies of Dapivirine and Clotrimazole were carried out by shaking an excess amount of each drug with 3 mL of the following dissolution medium simulated vaginal fluid, sodium phosphate buffer of pH 4 and 1% Tween, sodium phosphate buffer pH 4 and 0.5% sodium lauryl sulphate, sodium phosphate buffer pH4 and 1% sodium lauryl sulphate, sodium phosphate buffer pH 4 and methanol (ratio 75:25), sodium phosphate buffer pH4 and methanol (ratio 60:40), sodium phosphate buffer pH4 and methanol (ratio 40:60). (Gupta 2011) The vaginal pH of healthy women of reproductive age is acidic (pH 4-5). Hence sodium phosphate buffer (pH 4.0) was selected as the media. Dapivirine and Clotrimazole being poorly water soluble, solubility of these drugs were conducted in the selected media with different concentrations of surfactants and co solvents. The two media selected to study the release profile were sodium phosphate buffer containing 1) 1% SLS and 2) 60% methanol. (Gupta 2011) Liposome preparations (both suspensions and gels) were tested for <i>in vitro</i> stability in the phosphate buffer, pH 4.5. To be closer to human conditions, the same experiments were performed in the vaginal fluid simulant (VFS), pH 4.5. In order to develop a liposomal drug carrier system for localised and sustained vaginal delivery, it was necessary to check the stability of liposomes in conditions simulating vaginal environment. Since, healthy human vaginal mucous is characterised by pH ranging between 4.0 and 5.0 experiments were performed in the pH4.5 phosphate buffer. (Pavelić et al. 2005) The <i>in vitro</i> release of clotrimazole from chitosan microgranules was studied using as the dissolution medium 50 mL of acetate buffer (pH 5.2) with 1% SDS to maintain the sink condition. (Szymanska & Winnicka 2012) The release studies were performed with static Franz diffusion cells (0.9 cm in diameter, Crown Scientific, Sommerville, USA). The diffusion cells were thermoregulated with a water jacket at 32 °C. Cellulose nitrate (0.1µm pore diameter, Sartorius, Göttingen, Germany) membrane filters were mounted to Franz diffusion cells. Acetate buffer (pH 6.0) with 35% of dioxan was used as receptor fluid. 100 µl of SLN or NLC aqueous dispersion (containing 1% of clotrimazole) was applied to the donor compartment. (Souto et al. 2004) <i>In vitro</i> release studies were carried out using Spectra/Por Regenerated Cellulose Dialysis Membrane Tubes (12.000-14.000 MWCO). The membranes onto which the gel samples were placed were soaked in distilled water for 24 h before use. The chosen receptor medium was composed of phosphate buffer (pH 4.5, 65% v/v) and dioxane (35% v/v) and was continuously stirred during the release experiments. (Rençber et al. 2016) 	<ul style="list-style-type: none"> To evaluate CTZ permeation through the skin, samples of pig mucosa were mounted on standard Franz diffusion cells (area = 1.0 ± 0.1 cm²) and the receptor compartment (volume 11 mL) was filled with PBS. After equilibration, 1 mL of CTZ-NLC or CTZ-NLC-gel was added to the donor compartment. The receptor compartment was stirred at 250 rpm in a thermostatic water bath at a temperature of 37°C. (Ravani et al. 2013) <i>In vitro</i> drug permeation studies of clotrimazole in optimized liposomes/niosomes formulations and their corresponding gels were performed in the rabbit vaginal mucosa using vertical Franz diffusion cells. The receptor compartment had a volume of 7.2 mL and an effective diffusion area of 0.785 cm². The receptor compartment was filled with a PBS solution containing 10% (v/v) dioxane which was constantly stirred with a magnetic bar (37±1°C) throughout the experiments. (Ninga-b et al. 2005)
SER	<ul style="list-style-type: none"> Franz diffusion cell with an effective diffusion area of 2.8cm² was used for <i>ex vivo</i> permeation studies of HSM and commercial cream "Onabet". The skin samples were mounted carefully on diffusion cells and donor compartment consisting of test HSM. The receiver compartment was consisted of 30 mL ethanolic water to ensure sink condition and its temperature was maintained at 37±0.5°C with magnetic stirring at 600 rpm throughout the experiment. (Sahoo et al. 2016) In-vitro diffusion study of formulated bioadhesive gels was carried out on Franz diffusion cell having (Diameter of 1.5 cm with a diffusional area of 1.76 cm²). Rat abdominal skin was used as diffusion membrane. Pieces of rat abdominal skin membrane were soaked in phosphate buffer (PB) pH 6.8 for 8 hrs prior to experiment. Diffusion cell was filled with phosphate buffer pH 6.8 (Pande et al. 2014) Freshly excised porcine ear was obtained from a local abattoir no more than a few hours post-mortem and without any prior sanitization. The outer region of the skin was separated from the subcutaneous fat and stored at '80 °C until further use. Diffusion studies were performed using vertical Franz diffusion cells (Permegeare, Hellertown, PA, USA). The thawed skin was clamped between the donor and 	

	<p>receiver compartments of the diffusion cells. The temperature of the water bath was maintained at 37°C in order to ensure a skin surface temperature of 32°C. The receiver compartment contained PEG 400:1X PBS (phosphate buffered saline, pH 7.4) (60:40 v/v) which ensured sufficient solubility of the drug in order to maintain sink conditions. (Manian et al. 2016)</p> <ul style="list-style-type: none"> • The release rate of sertaconazole mucoadhesive vaginal tablet (n=3) was determined using The United States Pharmacopoeia (USP) XXIV dissolution testing apparatus I (basket method) in 500mL of phosphate buffer pH 4.0 as the dissolution medium. (Patel & Patel 2012a) • The <i>in vitro</i> drug release was, performed in sink conditions, by means of a Franz diffusion cell, diameter 20 mm, with water jacketed receptor chamber (15 mL) and a donor chamber thermostated at 37±0.5°C. The receptor solution was constantly stirred by magnetic stirrer. (Patel & Patel 2012b). • The release rate of sertaconazole effervescent mucoadhesive vaginal tablet (n=3) was determined using The United States Pharmacopoeia (USP) XXIV dissolution testing apparatus I (basket method) in 500mL of phosphate buffer pH 4.0 as the dissolution medium. (Anita Patel et al. n.d.) • The <i>in vitro</i> release of microsponges containing SN from the gel formulation was studied through cellophane membrane using Franz diffusion cell apparatus. The diffusion medium was freshly prepared phosphate buffer pH 6.8 and methanol (60:40) ratio was taken. (Pande et al. 2015) 	
ECO	<ul style="list-style-type: none"> • <i>In vitro</i> dissolution tests of pure drug and of the prevalent size fraction of microparticles were performed using a basket apparatus (Erweka DT600) rotating at 50rpm. As dissolution medium, 900mL of SVF were used at a temperature of 37°C. (Albertini et al. 2009) • The <i>in vitro</i> release studies of MN and EN from different chitosan gel bases were carried out using Spectra/Por Regenerated Cellulose Dialysis Membrane Tubes (12.000-14.000 MWCO). Membranes were soaked in distilled water for 24 hours before the experiment. Phosphate buffer (pH 4.5) with dioxane (65:35, V/V) was used as receptor medium. (Senyigit et al. 2014) • The drug release from eye gels and ocuserts, in phosphate buffer solution of pH 7.4 was carried out, using the dialysis method22. Spectra / Pore® dialysis membrane (12000-14000 molecular weight cutoffs) was soaked in phosphate buffer pH 7.4 for 24 hrs before the experiment. The membrane was stretched over the open end of 3 cm diameter glass tube and was made water tight by a rubber band. Two grams of each formulation were accurately weighed and thoroughly spreaded on the membrane. To each tube, 1.5 mL of the buffer solution pH 7.4 was added (Abd El-Gawad et al. 2016) • In-vitro release studies were carried out using Spectra/Por regenerated cellulose dialysis membrane tubes. The membranes onto which the gel samples were placed were soaked in distilled water for 24 h before use. The chosen receptor medium was composed of phosphate buffer (pH 4.5, 65% v/v) and dioxane (35% v/v), and was continuously stirred during the release experiments. (Baloglu et al. 2011) • The <i>in vitro</i> release of MEG formulations were studied using cellophane membrane using modified apparatus. The dissolution medium used was phosphate buffer, freshly prepared (pH 7.4). (Jaya Raja Kumar et al. 2015) 	No references
FEN	No references	No references
ISO	<ul style="list-style-type: none"> • The British Pharmacopoeia dissolution apparatus was used for the determination of release rates in this work. Each suppository was placed in a flask containing 150 mL Sorensen's phosphate buffer solution (pH 7.8). (Asikoglu et al. 1995) • <i>In vitro</i> release profiles of isoconazole nitrate from topical dosage forms (n = 3) were carried out using Franz-type vertical diffusion cell containing phosphate buffered (0,01M) pH 7.4 (7.0 mL) as acceptor phase and synthetic membrane (cellulose acetate with diffusion area of 1.77cm²). (Amaral et al.) 	No references

EST	<ul style="list-style-type: none"> The experiments were designed to mimic the normal “in-use” conditions in humans. These were conducted in volumetric 7-mL static vertical diffusion cells with automatic sampling (Microette Plus, Model 60-205–40; Hanson Research, Chatsworth, California). The skin discs were positioned between the donor and the receptor compartments with the stratum corneum (SC) uppermost, ensuring that all air under the skin was removed. The available diffusion area was 1.86 cm² and a clamp was used to hold the compartments together. The receptor chamber was filled with PBS pH 7.4 + 0.5% HPCD, stirred at 600 rpm and maintained at 32°C ± 2°C during the whole experiment. (Brandão et al. 2014) <i>In vitro</i> release tests were performed in 7-mL static vertical diffusion cells with automatic sampling (Microette Plus®, Hanson Research, USA). The donor compartment contained the hormone formulations (n = 6 for each formulation), and the receptor compartment was filled with the respective receptor medium, ensuring that air under the artificial membrane was completely eliminated. Polysulfone membrane disc filters, 25 mm diameter (Tuffryn®, Pall Corporation, USA), were rinsed to remove any additives that prevented drug release from the formulations and were positioned between the cell compartments. An infinite dose (300 mg) of each formulation was applied to the membrane surface using a calibrated positive displacement pipette Pos-D MR-110 (Rainin, USA), which prevented solvent evaporation and reduced any compositional change. The emulsions were then carefully spread to achieve complete uniform coverage, with a diffusion area of 1.86 cm², with the compartments held together using a clamp. The receptor medium was continuously mixed using a magnetic stirring bar (300 rpm, 32 ± 2 °C during the entire measurement), except during the sample collecting period. Aliquots (1 mL) were withdrawn at regular time intervals (0.5, 1, 2, 3, 4, 8, 12, 16, 20, and 24 h), collected into HPLC vials, and immediately replaced with the receptor medium at the same temperature. The hormone concentrations were correspondingly corrected for the replenishments. (Polonini et al. 2014) 	No references
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